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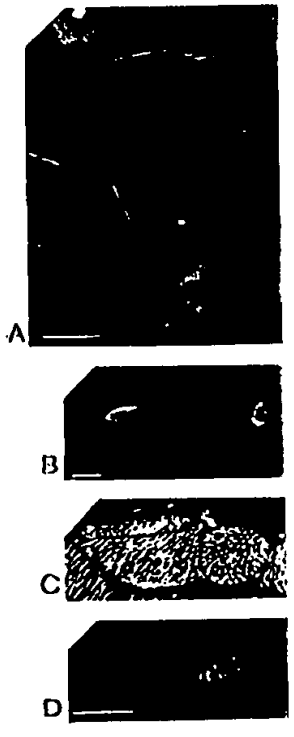
(54) Title: USE OF NEURONAL APOPTOSIS INHIBITOR PROTEIN (NAIP)

(57) Abstract

The invention provides NAIP nucleic acid and sequences. Also provided are anti-NAIP antibodies and methods for modulating apoptosis and detecting compounds which modulate apoptosis.



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USE OF NEURONAL APOPTOSIS INHIBITOR PROTEIN (NAIP)

Field of the Invention

This invention relates in general to the function of the NAIP inhibitor protein in apoptosis and more particularly to the use of NAIP antibodies, proteins, and nucleic acids to characterize NAIP, identify compounds which modulate NAIP, and diagnose and treat conditions affected by changes in NAIP levels.

Background of the Invention

Apoptosis is a morphologically distinct form of programmed cell death that is important in the normal development and maintenance of multicellular organisms. Dysregulation of apoptosis can take the form of inappropriate suppression of cell death, as occurs in the development of some cancers, or in a failure to control the extent of cell death, as is believed to occur in acquired immunodeficiency and certain neurodegenerative disorders, such as spinal muscular atrophy (SMA).

Childhood spinal muscular atrophies are neurodegenerative disorders characterized by progressive spinal cord motor neuron depletion and are among the most common autosomal recessive disorders (Dubowitz, V. 1978, Brooke, M.A. 1986). Type I SMA is the most frequent inherited cause of death in infancy. The loss of motor neurons in SMA, has led to suggestions that an inappropriate continuation or reactivation of normally occurring motor neuron apoptosis may underlie the disorder (Sarnat, H.B. 1992). NAIP, a gene associated with SMA, has been mapped to human chromosome 5q13.1

Some baculoviruses encode proteins that are termed inhibitors of apoptosis proteins (IAPs) because they inhibit the apoptosis that would otherwise occur when insect cells are infected by the virus. These proteins are thought to work in a manner that is independent of other viral proteins. The baculovirus IAP genes include sequences encoding a ring zinc finger-like motif (RZF), which may be involved in DNA binding, and two N-terminal domains that consist of a 70 amino acid repeat motif termed a BIR domain (Baculovirus IAP Repeat).

Summary of the Invention

We have discovered uses for NAIP proteins, nucleic acids, and antibodies for the detection and treatment of conditions involving apoptosis. Furthermore, we have discovered a novel NAIP sequence and a NAIP fragment with enhanced anti-apoptotic activities.

In general, the invention features a substantially pure nucleic acid molecule, such as a genomic, cDNA, antisense DNA, RNA, or a synthetic nucleic acid molecule, that encodes or corresponds to a mammalian NAIP polypeptide. This nucleic acid may be incorporated into a vector. Such a vector may be in a cell, such as a mammalian, yeast, nematode, or bacterial cell. The nucleic acid may also be incorporated into a transgenic animal or embryo thereof. In preferred embodiments, the nucleic acid molecule is a human NAIP nucleic acid. In most preferred embodiments the NAIP gene is a human NAIP gene. In other various preferred embodiments, the cell is a transformed cell.

According to one preferred embodiment, the nucleic acid sequence includes the cDNA sequences encoding exons 14a and 17. In a more preferred embodiment the sequence includes exons 1-14, 14a, and 15-17. In the most preferred embodiments the sequence also includes the complete 5' and 3' untranslated regions of the NAIP gene and is represented as Seq. ID No. 2, 21, or 23, most preferably, as in Seq. ID No. 21. In other preferred embodiments, the nucleic acid is a purified nucleotide sequence comprising genomic DNA, cDNA, mRNA, anti-sense DNA or other DNA substantially identical to the cDNA sequences of Seq. ID No. 2, 21, or 23 corresponding to the cDNA sequences of the invention. Most preferably exons 1 to 14 and 14a to 17 are as described in Seq. ID No. 21.

In specific embodiments, the invention features nucleic acid sequences substantially identical to the sequences shown in Fig. 21, or fragments thereof. In another aspect, the invention also features RNA which is encoded by the DNA described herein. Preferably, the RNA is mRNA. In another embodiment the RNA is antisense RNA that is complementing to the coding strand of NAIP.

In a second aspect of the invention, the NAIP encoding nucleic acid comprises at least the 3 BIR domains of a NAIP sequence provided herein (e.g., nucleotides 1-1360 of the NAIP sequence provided in Fig. 6), but lacks at least some of the sequences encoding the carboxy

terminus of the NAIP polypeptide. Preferably, at least 30 nucleic acids are deleted from the region of the NAIP gene between nucleic acids 1360 (i.e., the end of the BIR domains) 4607 (i.e., the end of the coding sequence) of the NAIP sequence shown in Fig. 6, Seq. ID No. 21. More preferably, at least 100 nucleotides are deleted, and even more preferably at least 1000 nucleotides are deleted. In the most preferred embodiment, up to 3247 nucleotides are deleted. Preferably, the deletion results in a statistically significant increase in the anti-apoptotic activity of the encoded protein on one of the assays provided herein.

In a third aspect, the invention features a substantially pure DNA which includes a promoter capable of expressing or activating the expression of the NAIP gene or fragments thereof in a cell susceptible to apoptosis. In preferred embodiments of this aspect, the NAIP gene is human NAIP or fragments thereof, as described above. In further preferred embodiments of this aspect of the invention, the promoter is the promoter native to the NAIP gene. Additionally, transcriptional and translational regulatory regions are, preferably, those native to a NAIP gene.

In another aspect, the invention provides transgenic cell lines, including the NAIP nucleic acids of the invention. The transgenic cells of the invention are preferably cells that are altered in their apoptotic response. In preferred embodiments, the transgenic mammalian cell is a fibroblast, neuronal cell, a pulmonary cell, a renal cell, a lymphocyte cell, a glial cell, a myocardial cell, an embryonic stem cell, or an insect cell. Most preferably, the neuron is a motor neuron and the lymphocyte is a $CD4^+$ T cell.

In another related aspect, the invention features a method of altering the level of apoptosis that involves producing a transgenic cell having a transgene encoding a NAIP polypeptide or antisense nucleic acid. The transgene is integrated into the genome of the cell in a way that allows for expression. Furthermore, the level of expression in the cell is sufficient to alter the level of apoptosis. In preferred embodiments the transgene is in a motor neuron or a myocardial cell.

In yet another related aspect, the invention features a transgenic animal, preferably a mammal, more preferably a rodent, and most preferably a mouse, having a NAIP gene as described above inserted into the genome (mutant or wild-type), or a knockout of a NAIP gene in

the genome, or both. A transgenic animal expressing NAIP antisense nucleic acid is also included. The transgenic animals may express either an increased or a decreased amount of NAIP polypeptide, depending on the construct used and the nature of the genomic alteration. For example, utilizing a nucleic acid molecule that encodes all or part of a NAIP to engineer a knockout mutation in a NAIP gene would generate an animal with decreased expression of either all or part of the corresponding NAIP polypeptide. In contrast, inserting exogenous copies of all or part of a NAIP gene into the genome, preferably under the control of active regulatory and promoter elements, would lead to increased expression or the corresponding NAIP polypeptide.

In another aspect, the invention features a method of detecting a NAIP gene in a cell by detecting the NAIP gene, or a portion thereof (which is greater than 9 nucleotides, and preferably greater than 18 nucleotides in length), with a preparation of genomic DNA from the cell. The NAIP gene and the genomic DNA are brought into contact under conditions that allow for hybridization (and therefore, detection) of nucleic acid sequences in the cell that are at least 50% identical to the DNA encoding the NAIP polypeptides. Preferably, the nucleic acid used comprised at least a part of exon 14a or exon 17, as provided in Figs. 6 and 7.

In another aspect, the invention features a method of producing a NAIP polypeptide *in vivo* or *in vitro*. In one embodiment, this method involves providing a cell with nucleic acid encoding all or part of a NAIP polypeptide (which is positioned for expression in the cell), culturing the cell under conditions that allow for expression of the nucleic acid, and isolating the NAIP polypeptide. In preferred embodiments, the NAIP polypeptide is expressed by DNA that is under the control of a constitutive or inducible promotor. As described herein, the promotor may be a native or heterologous promotor. In preferred embodiments the nucleic acid comprises exon 14a or exon 17. Most preferably the nucleic acid is the nucleic acid shown in either Fig. 6 or Fig. 7. Most preferably, it is the sequence of Fig. 6.

In another aspect, the invention features substantially pure mammalian NAIP polypeptide. Preferably, the polypeptide includes an amino acid sequence that is substantially identical to one of the amino acid sequences shown in any one of Figs. 6 or 7. Most preferably, the polypeptide is the human NAIP polypeptide of Fig. 6. Fragments including at least two BIR domains, as provided herein, are also a part of the invention. Preferably, the fragment has at least

three BIR domains. For example, polypeptides encoded by the nucleic acids described above having deletions between nucleic acids 1360 and the end of the gene are a part of the invention. In one embodiment, the NAIP fragments included those NAIP fragments comprising at least 15 sequential amino acids of Seq. ID No. 22 or 24. Most preferably the fragment includes at least a portion of exon 14a or exon 17.

In another aspect, the invention features a recombinant mammalian polypeptide derived from NAIP that is capable of modulating apoptosis. The polypeptide may include at least two BIR domains as defined herein, preferably three BIR domains. In preferred embodiments, the NAIP amino acid sequence differs from the NAIP sequences of Figs. 6 or 7 by only conservative substitutions or differs from the sequences encoded by the nucleic acids of Seq. ID Nos. 1, 2, 21 or 23 by deletions of amino acids carboxy terminal to the BIR domains. In other preferred embodiments the recombinant protein decreases apoptosis relative to a control by at least 5%, more preferably by 25%.

In another aspect, the invention features a method of inhibiting apoptosis in a mammal wherein the method includes: providing nucleic acid encoding a NAIP polypeptide to a cell that is susceptible to apoptosis; wherein the nucleic acid is positioned for expression in the cell; NAIP gene is under the control of regulatory sequences suitable for controlled expression of the gene(s); and the NAIP transgene is expressed at a level sufficient to inhibit apoptosis relative to a cell lacking the NAIP transgene. The nucleic acid may encode all or part of a NAIP polypeptide. It may, for example, encode two or three BIR domains, but have a deletion of the carboxy-terminal amino acids. Preferably, the nucleic acid comprises sequences encoding exon 14a, exon 17, or both.

In a related aspect, the invention features a method of inhibiting apoptosis by producing a cell that has integrated, into its genome, a transgene that includes the NAIP gene, or a fragment thereof. The NAIP gene may be placed under the control of a promoter providing constitutive expression of the NAIP gene. Alternatively, the NAIP transgene may be placed under the control of a promoter that allows expression of the gene to be regulated by environmental stimuli. For example, the NAIP gene may be expressed using a tissue-specific or cell type-specific promoter, or by a promoter that is activated by the introduction of an external signal or agent, such as a

chemical signal or agent. In preferred embodiments the mammalian cell is a lymphocyte, a neuronal cell, a glial cell, or a fibroblast. In other embodiments, the cell in an HIV-infected human, or in a mammal suffering from a neurodegenerative disease, an ischemic injury, a toxin-induced liver disease, or a myelodysplastic syndrome.

In a related aspect, the invention provides a method of inhibiting apoptosis in a mammal by providing an apoptosis-inhibiting amount of NAIP polypeptide. The NAIP polypeptide may be a full-length polypeptide, or it may be one of the fragments described herein.

In another aspect, the invention features a purified antibody that binds specifically to a NAIP protein. Such an antibody may be used in any standard immunodetection method for the detection, quantification, and purification of a NAIP polypeptide. Preferably, the antibody binds specifically to NAIP. The antibody may be a monoclonal or a polyclonal antibody and may be modified for diagnostic or for therapeutic purposes. The most preferable antibody binds the NAIP polypeptide sequences of Seq. ID Nos. 22 and/or 24, but not the NAIP polypeptide sequence disclosed in PCT/CA95/00581.

The antibodies of the invention may be prepared by a variety of methods. For example, the NAIP polypeptide, or antigenic fragments thereof, can be administered to an animal in order to induce the production of polyclonal antibodies. Alternatively, antibodies used as described herein may be monoclonal antibodies, which are prepared using hybridoma technology (see, e.g., Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, 1981). The invention features antibodies that specifically bind human or murine NAIP polypeptides, or fragments thereof. In particular, the invention features "neutralizing" antibodies. By "neutralizing" antibodies is meant antibodies that interfere with any of the biological activities of the NAIP polypeptide, particularly the ability of NAIP to inhibit apoptosis. The neutralizing antibody may reduce the ability of NAIP polypeptides to inhibit apoptosis by, preferably 50%, more preferably by 70%, and most preferably by 90% or more. Any standard assay of apoptosis, including those described herein, may be used to assess potentially neutralizing antibodies.

In addition to intact monoclonal and polyclonal anti-NAIP antibodies, the invention features various genetically engineered antibodies, humanized antibodies, and antibody fragments, including F(ab')2, Fab', Fab, Fv and sFv fragments. Antibodies can be humanized by methods known in the art, e.g., monoclonal antibodies with a desired binding specificity can be commercially humanized (Scotgene, Scotland; Oxford Molecular, Palo Alto, CA). Fully human antibodies, such as those expressed in transgenic animals, are also features of the invention (Green et al., Nature Genetics 7:13-21, 1994).

Ladner (U.S. Patent 4,946,778 and 4,704,692) describes methods for preparing single polypeptide chain antibodies. Ward et al. (Nature 341:544-546, 1989) describe the preparation of heavy chain variable domains, which they term "single domain antibodies," which have high antigen-binding affinities. McCafferty et al. (Nature 348:552-554, 1990) show that complete antibody V domains can be displayed on the surface of fd bacteriophage, that the phage bind specifically to antigen, and that rare phage (one in a million) can be isolated after affinity chromatography. Boss et al. (U.S. Patent 4,816,397) describe various methods for producing immunoglobulins, and immunologically functional fragments thereof, which include at least the variable domains of the heavy and light chain in a single host cell. Cabilly et al. (U.S. Patent 4,816,567) describe methods for preparing chimeric antibodies.

In another aspect, the invention features a method of identifying a compound that modulates apoptosis. The method includes providing a cell expressing or capable of expressing a NAIP polypeptide, contacting the cell with a candidate compound, and monitoring the expression of the NAIP gene or a reporter gene linked to the NAIP gene regulatory sequences, or by monitoring NAIP biological activity. An alteration in the level of expression of the NAIP gene indicates the presence of a compound which modulates apoptosis. The compound may be an inhibitor or an enhancer of apoptosis. In various preferred embodiments, the mammalian cell is a myocardial cell, a fibroblast, a neuronal cell, a glial cell, a lymphocyte (T cell or B cell), or an insect cell.

In a related aspect, the invention features methods of detecting compounds that modulate apoptosis using the interaction trap technology and NAIP polypeptides, or fragments thereof, as a

component of the bait. In preferred embodiments, the compound being tested as a modulator of apoptosis is also a polypeptide.

In a related aspect, the invention features a method for analyzing the anti-apoptotic effect of a candidate NAIP is provided comprising, i) providing an expression vector for the expression of the candidate NAIP; ii) transfecting mammalian cells with said expression vector; iii) inducing the transformed cells to undergo apoptosis; and iv) comparing the survival rate of the cells with appropriate mammalian cell controls.

In yet another aspect, the invention features a method for detecting the expression of NAIP in tissues comprising, i) providing a tissue or cellular sample; ii) incubating said sample with an anti-NAIP polyclonal or monoclonal antibody; and iii) visualizing the distribution of NAIP.

In another aspect, the invention features a method for diagnosing a cell proliferation disease, or an increased likelihood of such a disease, using a NAIP nucleic acid probe or NAIP antibody. Preferably, the disease is a cancer of the central nervous system. Most preferably, the disease is selected from the group consisting of neuroblastoma, meningioma, glialblastoma, astracystoma, neuroastrocytoma, promyelocytic leukemia, a HeLa-type carcinoma, chronic myelogenous leukemia (preferably using xiap or hiap-2 related probes), lymphoblastic leukemia (preferably using a xiap related probe), Burkitt's lymphoma, colorectal adenocarcinoma, lung carcinoma, and melanoma. Preferably, a diagnosis is indicated by a 2-fold increase in expression or activity, more preferably, at least a 10-fold increase in expression or activity.

In another aspect, the invention includes a method of treating a patient having deleterious levels apoptosis. Where the patient has more apoptosis than desirable or is otherwise deficient in normal NAIP, the method includes the step of administering to said patient a therapeutically effective amount of NAIP protein, NAIP nucleic acid, or a compound which enhances NAIP activity levels in a form which allows delivery to the cells which are undergoing more apoptosis than is therapeutically desirable. In one preferred embodiment, the cell having deleterious levels of apoptosis is a myocardial cell in a patient diagnosed with a cardiac condition.

Where insufficient levels of apoptosis are likely to occur, antisense NAIP nucleic acid, NAIP antibody, or a compound which otherwise decreases NAIP activity levels may be

administered. Treatment of SMA is specifically excluded from the invention. Thus, apoptosis may be induced in a cell by administering to the cell a negative regulator of the NAIP-dependent anti-apoptotic pathway. The negative regulator may be, but is not limited to, a NAIP polypeptide fragment or purified NAIP specific antibody. For example, the antibody may bind to an epitope in any one of the three BIR domains. The negative regulator may also be a NAIP antisense RNA molecule.

Skilled artisans will recognize that a mammalian NAIP, or a fragment thereof (as described herein), may serve as an active ingredient in a therapeutic composition. This composition, depending on the NAIP or fragment included, may be used to modulate apoptosis and thereby treat any condition that is caused by a disturbance in apoptosis. Thus, it will be understood that another aspect of the invention described herein, includes the compounds of the invention in a pharmaceutically acceptable carrier.

As summarized above, a NAIP nucleic acid, polypeptide, or antibody may be used to modulate apoptosis. Furthermore, a NAIP nucleic acid, polypeptide, or antibody may be used in the discovery and/or manufacture of a medicament for the modulation of apoptosis.

By "NAIP gene" is meant a gene encoding a polypeptide having at least exon 14a or exon 17 Figs. 6 or 7, or the sequence of Fig. 5, Seq. ID No. 1, wherein at least 10 carboxy-terminal nucleic acids have been deleted to enhance activity, as described above. In preferred embodiments the NAIP gene encodes a polypeptide which is capable of inhibiting apoptosis or eliciting antibodies which specifically bind NAIP. In preferred embodiments the NAIP gene is a gene having about 50% or greater nucleotide sequence identity to the NAIP amino acid encoding sequences of Figs. 6 or 7. In another preferred embodiment, the NAIP gene encodes a fragment sufficient to inhibit apoptosis. Preferably, the region of sequence over which identity is measured is a region encoding exon 14a or exon 17. Mammalian NAIP genes include nucleotide sequences isolated from any mammalian source. Preferably, the mammal is a human.

The term "NAIP gene" is meant to encompass any NAIP gene, which is characterized by its ability to modulate apoptosis and encodes a polypeptide that has at least 20%, preferably at least 30%, and most preferably at least 50% amino acid sequence identity with the NAIP

polypeptides shown in Figs. 6 and 7. Specifically excluded is the full length sequence disclosed in PCT/CA95/00581 and shown in Seq. ID No. 1.

By "NAIP protein" or "NAIP polypeptide" is meant a polypeptide, or fragment thereof, encoded by a NAIP gene as described above.

By "modulating apoptosis" or "altering apoptosis" is meant increasing or decreasing the number of cells that would otherwise undergo apoptosis in a given cell population. Preferably, the cell population is selected from a group including T cells, neuronal cells, fibroblasts, myocardial cells, or any other cell line known to undergo apoptosis in a laboratory setting (e.g., the baculovirus infected insect cells). It will be appreciated that the degree of modulation provided by a NAIP or a modulating compound in a given assay will vary, but that one skilled in the art can determine the statistically significant change in the level of apoptosis which identifies a NAIP or a compound which modulates a NAIP.

By "inhibiting apoptosis" is meant any decrease in the number of cells which undergo apoptosis relative to an untreated control. Preferably, the decrease is at least 25%, more preferably the decrease is 50%, and most preferably the decrease is at least one-fold.

By "polypeptide" is meant any chain of more than two amino acids, regardless of post-translational modification such as glycosylation or phosphorylation.

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 50%, preferably 85%, more preferably 90%, and most preferably 95% homology to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative

substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "substantially pure polypeptide" is meant a polypeptide that has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is a NAIP polypeptide that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure NAIP polypeptide may be obtained, for example, by extraction from a natural source (e.g. a fibroblast, neuronal cell, or lymphocyte) by expression of a recombinant nucleic acid encoding a NAIP polypeptide, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes. By "substantially pure DNA" is meant DNA that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) a NAIP polypeptide.

By "transgene" is meant any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell which includes a DNA sequence which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic mammalian (e.g., rodents such as rats or mice) and the DNA (transgene) is inserted by artifice into the nuclear genome.

By "transformation" is meant any method for introducing foreign molecules into a cell. Lipofection, calcium phosphate precipitation, retroviral delivery, electroporation, and biolistic transformation are just a few of the teachings which may be used. For example, biolistic transformation is a method for introducing foreign molecules into a cell using velocity driven microprojectiles such as tungsten or gold particles. Such velocity-driven methods originate from pressure bursts which include, but are not limited to, helium-driven, air-driven, and gunpowder-driven techniques. Biolistic transformation may be applied to the transformation or transfection of a wide variety of cell types and intact tissues including, without limitation, intracellular organelles (e.g., and mitochondria and chloroplasts), bacteria, yeast, fungi, algae, animal tissue, and cultured cells.

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., a NAIP polypeptide, a recombinant protein or a RNA molecule).

By "reportor gene" is meant a gene whose expression may be assayed; such genes include, without limitation, glucuronidase (GUS), luciferase, chloramphenicol transacetylase (CAT), and β-galactosidase, and green fluorescent protein (GFP).

By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the native gene

By "operably linked" is meant that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins are bound to the regulatory sequences).

By "conserved region" is meant any stretch of six or more contiguous amino acids exhibiting at least 30%, preferably 50%, and most preferably 70% amino acid sequence identity between two or more of the NAIP family members, (e.g., between human NAIP and murine NAIP).

By "carboxy terminal amino acids of NAIP" is meant the amino acids of carboxy terminal to the three BIR domains of the NAIP gene. For example, the amino acids encoded beyond nucleic acid 1360 of Seq. ID. No. 21 are carboxy terminal.

By "detectably-labelled" is meant any means for marking and identifying the presence of a molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, or a cDNA molecule. Methods for detectably-labelling a molecule are well known in the art and include, without limitation, radioactive labelling (e.g., with an isotope such as ^{32}P or ^{35}S) and nonradioactive labelling (e.g., chemiluminescent labelling, e.g., fluorescein labelling).

By "antisense," as used herein in reference to nucleic acids, is meant a nucleic acid sequence, regardless of length, that is complementary to the coding strand of a gene.

By "purified antibody" is meant antibody which is at least 60%, by weight, free from proteins and naturally occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably 90%, and most preferably at least 99%, by weight, antibody, e.g., a NAIP specific antibody. A purified antibody may be obtained, for example, by affinity chromatography using recombinantly-produced protein or conserved motif peptides and standard techniques.

By "specifically binds" is meant an antibody that recognizes and binds a protein but that does not substantially recognize and bind other molecules in a sample, e.g., a biological sample, that naturally includes protein. The preferred antibody binds to the NAIP peptide sequence of sequence ID No. 2 but does not bind to the NAIP sequence disclosed in PCT/CA 95/00581.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Various aspects of the invention are described with respect to the drawings wherein:

Fig. 1. shows expression of NAIP in HeLa, CHO and Rat-1 pooled stable lines and adenovirus infected cells analysed by Western blotting (A-D) and immunofluorescence. A-B are cells infected with adenovirus encoding NAIP-myc detected by a mouse anti-myc monoclonal antibody or by a rabbit anti human NAIP polyclonal antibody. C cells infected with adenovirus encoding NAIP detected by the NAIP polyclonal antibody. D expression of myc-NAIP in representative pooled cell lines by immunofluorescence detected with antibodies against myc. E-F rat-1 NAIP transfectants detected by E anti-myc and F anti-NAIP antibodies.

Fig. 2. shows the effect of NAIP on cell death induced by serum deprivation, menadione and TNF-α. Viability of a CHO cells deprived of serum in A, adenovirus infected cells and B, pooled transformants. C-H, cell death induced by menadione in adenvirus infected CHO (C, D) and Rat-1 (E, F and G, H) adenovirus infected cells and pooled transformants respectively. I, adenovirus infected and J, pooled transformants of TNF-α/cyclohexamide treated HeLa cells.

Fig. 3. shows immunofluorescence analysis of human spinal cord tissue. A, Anterior horn cells. B, Intermediolateral neurons. C, Dorsal roots. D, Ventral roots.

Fig. 4. depicts the genomic structure of PAC 125D9 from human chromosome 5q13.1. Both strands of the 131,708 bp region shown in the figure have been sequenced and can be found as GenBank accession #U80017. NotI (N), EcoRI (E), HindIII (H) and BamHI (B) sites are indicated. The exons of BTF2p44 **(green)**, NAIP **(red)** and SMN **(grey)** are represented above by numbered color boxes. The transcribed (but not translated) CCA sequence is indicated by the light green box. The number of nucleotides which a specific region spans is as indicated, e.g. the gap between NAIP and SMN is 15471 bp. The minimal tiling pattern of plasmid clones covering the PAC is shown below. The letters at the beginning of each clone indicate the restriction enzymes used for preparing the plasmid libraries, except for 1C6, 2A8 and 2E2 which are clones from the partial Sau3AI libraries. (SstI-S). The location and orientation of eight classes of repeat sequences found using the NIH Sequin program are depicted by **color** triangles . The names of the repeats represented by **different colors** are shown at the top right of the figure. Promotor sequences as detected by GRAIL

(red arrow) or Prestridge (Prestidge, D. S. *J.Mol. Biol.* 249, 923-932 (1995) (green arrow) programs and CpG islands are shown as arrows or blue blocks respectively above the bar.

Fig. 5 shows the sequences obtained in 2 separate sequencings of the NAIP gene.

Fig. 6 shows a preferred NAIP cDNA sequence and the predicted NAIP polypeptide sequence.

Fig. 7 shows a NAIP sequence including the intron-exon boundaries. (Seq. ID No. 23).

Detailed Description of the Preferred Embodiment

Although the precise site and mechanism of NAIP's anti-apoptotic effect is unknown, it is now demonstrated that NAIP is clearly involved in apoptotic pathways in mammalian cells. In addition, immunofluorescence localization indicates that NAIP is expressed in motor, but not sensory neurons. These findings are in keeping with the protein acting as a negative regulator of apoptosis, most particularly neuronal apoptosis and, when deficient or absent, contributes to the neurodegenerative phenotypes such as SMA and ALS.

I. The NAIP gene

There are two nearly identical copies of NAIP on 5q13.1. The complete NAIP gene, shown in Fig. 6, contains 18 exons (1 to 14, and 14a to 17) and spans an estimated 90 kb of genomic DNA. (Other intermediate sequences obtained are shown in Figs. 5 and 7). The NAIP coding region spans 4212 nucleotides resulting in a predicted gene product of 1404 amino acids (Seq. ID No. 22). The total length of the NAIP gene spans 6228 nucleotides (Seq. ID No. 21) with a 395 nucleotide 5' UTR and a 1621 nucleotide 3' UTR. The complete sequence, Sequence ID No.2, allows one skilled in the art to develop probes and primers for the identification of homologous sequences and for the identification of mutations within the DNA. Both 5' and 3' regions may also prove useful as encoding binding sites for agents which may up or down-regulate the gene further delineating the NAIP pathway and function. The sequences identified as Seq. ID No. 2 and 23 are also useful for protein expression in appropriate vectors and hosts to produce NAIP and study its function as well

as to develop antibodies. Sequencing of the PAC 125D9 154 kb, which was identified as a likely site of the SMA gene, resulted in the identification of the NAIP sequence shown in Fig. 5, Seq. ID No. 1. An additional coding sequence, exon 14a, has since been identified and is provided herewith. The NAIP DNA sequence containing exon 14a appears to be a predominant gene isoform which is not deleted or mutated in SMA patients. The techniques and primers used for the isolation and application of exon 14a from the human fetal spinal cord cDNA libraries was as described for the identification of the other exons and detailed in Example 4. Additional screening of cDNA libraries combined with analysis of PAC 125D9 genomic DNA sequence has resulted in the identification of a novel 3' end of NAIP which includes additional exon 17 sequence.

II. Synthesis of NAIP

The characteristics of the cloned NAIP gene sequence may be analyzed by introducing the sequence into various cell types or using *in vitro* extracellular systems. The function of the NAIP may then be examined under different physiological conditions. The NAIP DNA sequence may be manipulated in studies to understand the expression of the gene and gene product. Alternatively, cell lines may be produced which overexpress the gene product allowing purification of NAIP for biochemical characterization, large-scale production, antibody production, and patient therapy.

For protein expression, eukaryotic and prokaryotic expression systems may be generated in which the NAIP gene sequence is introduced into a plasmid or other vector which is then introduced into living cells. Constructs in which the NAIP cDNA sequence containing the entire open reading frame inserted in the correct orientation into an expression plasmid may be used for protein expression. Alternatively, portions of the sequence, including wild-type or mutant NAIP sequences, may be inserted. Prokaryotic and eukaryotic expression systems allow various important functional domains of the protein to be recovered as fusion proteins and then used for binding, structural and functional studies and also for the generation of appropriate antibodies. If a NAIP increases apoptosis, it may be desirable to express that protein under control of an inducible promotor.

Typical expression vectors contain promoters that direct the synthesis of large amounts of mRNA corresponding to the gene. They may also include sequences allowing for their autonomous replication within the host organism, sequences that encode genetic traits that allow cells containing the vectors to be selected, and sequences that increase the efficiency with which the mRNA is translated. Some vectors contain selectable markers such as neomycin resistance that permit isolation of cells by growing them under selective conditions. Stable long-term vectors may be maintained as freely replicating entities by using regulatory elements of viruses. Cell lines may also be produced which have integrated the vector into the genomic DNA and in this manner the gene product is produced on a continuous basis.

Expression of foreign sequences in bacteria such as *E.coli* require the insertion of the NAIP sequence into an expression vector, usually a bacterial plasmid. This plasmid vector contains several elements such as sequences encoding a selectable marker that assures maintenance of the vector in the cell, a controllable transcriptional promoter (*ie,* lac) which upon induction can produce large amounts of mRNA from the cloned gene, translational control sequences and a polylinker to simplify insertion of the gene in the correct orientation within the vector. In a simple *E. coli* expression vector utilizing the lac promoter, the expression vector plasmid contains a fragment of the *E.coli* chromosome containing the lac promoter and the neighboring lacZ gene. In the presence of the lactose analog IPTG, RNA polymerase normally transcribes the lacZ gene producing lacZ mRNA which is translated into the encoded protein, β-galactosidase. The lacZ gene can be cut out of the expression vector with restriction enzymes and replaced by NAIP gene sequence. When this resulting plasmid is transfected into *E.coli*, addition of IPTG and subsequent transcription from the lac promoter produces NAIP mRNA, which is translated into NAIP.

Once the appropriate expression vector containing the NAIP gene is constructed it is introduced into an appropriate *E.coli* strain by transformation techniques including calcium phosphate transfection, DEAE-dextran transfection, electroporation, microinjection, protoplast fusion and liposome-mediated transfection.

The host cell which may be transfected with the vector of this invention may be selected from the group consisting of *E.coli*, *pseudomonas*, *bacillus subtillus*, or other *bacili*, other bacteria, yeast, fungi, insect (using baculoviral vectors for expression), mouse or other animal or human tissue cells. Mammalian cells can also be used to express the NAIP protein using a vaccinia virus expression system.

In vitro expression of proteins encoded by cloned DNA is also possible using the T7 late-promoter expression system. This system depends on the regulated expression of T7 RNA polymerase which is an enzyme encoded in the DNA of bacteriophage T7. The T7 RNA polymerase transcribes DNA beginning within a specific 23-bp promotor sequence called the T7 late promoter. Copies of the T7 late promoter are located at several sites on the T7 genome, but none is present in *E.coli* chromosomal DNA. As a result, in T7 infected cells, T7 RNA polymerase catalyzes transcription of viral genes but not of *E.coli* genes. In this expression system recombinant *E.coli* cells are first engineered to carry the gene encoding T7 RNA polymerase next to the lac promoter. In the presence of IPTG, these cells transcribe the T7 polymerase gene at a high rate and synthesize abundant amounts of T7 RNA polymerase. These cells are then transformed with plasmid vectors that carry a copy of the T7 late promoter protein. When IPTG is added to the culture medium containing these transformed *E.coli cells*, large amounts of T7 RNA polymerase are produced. The polymerase then binds to the T7 late promoter on the plasmid expression vectors, catalyzing transcription of the inserted cDNA at a high rate. Since each *E.coli* cell contains many copies of the expression vector, large amounts of mRNA corresponding to the cloned cDNA can be produced in this system and the resulting protein can be radioactively labelled. Plasmid vectors containing late promoters and the corresponding RNA polymerases from related bacteriophages such as T3, T5 and SP6 may also be used for *in vitro* production of proteins from cloned DNA. *E.coli* can also be used for expression by infection with M13 Phage mGPI-2. *E.coli* vectors can also be used with phage lambda regulatory sequences, by fusion protein vectors, by maltose-binding protein fusions, and by glutathione-S-transferase fusion proteins.

A preferred expression system is the baculovirus system using, for example, the vector pBacPAK9, which is available from Clontech (Palo Alto, CA). If desired, this system may be used

in conjunction with other protein expression techniques, for example, the myc tag approach described by Evan et al. (Mol. Cell Biol. 5:3610-3616, 1985).

Eukaryotic expression systems permit appropriate post-translational modifications to expressed proteins. This allows for studies of the NAIP gene and gene product including determination of proper expression and post-translational modifications for biological activity, identifying regulatory elements located in the 5' region of the NAIP gene and their role in tissue regulation of protein expression. It also permits the production of large amounts of normal and mutant proteins for isolation and purification, to use cells expressing NAIP as a functional assay system for antibodies generated against the protein, to test the effectiveness of pharmacological agents or as a component of a signal transduction system, to study the function of the normal complete protein, specific portions of the protein, or of naturally occurring polymorphisms and artificially produced mutated proteins. The NAIP DNA sequence can be altered using procedures such as restriction enzyme digestion, DNA polymerase fill-in, exonuclease deletion, terminal deoxynucleotide transferase extension, ligation of synthetic or cloned DNA sequences and site-directed sequence alteration using specific oligonucleotides together with PCR.

A NAIP may be produced by a stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Pouwels et al. (*supra*), as are methods for constructing such cell lines (see e.g., Ausubel et al. (*supra*). In one example, cDNA encoding a NAIP is cloned into an expression vector that includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, integration of the NAIP-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300 μM methotrexate in the cell culture medium (as described, Ausubel et al., *supra*). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene.

Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (*supra*). These methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. The most commonly used DHFR-containing expression vectors

are pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., *supra*). The host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR$^-$ cells, ATCC Accession No. CRL 9096) are among those most preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

Once the recombinant protein is expressed, it is isolated by, for example, affinity chromatography. In one example, an anti-NAIP antibody, which may be produced by the methods described herein, can be attached to a column and used to isolate the NAIP protein. Lysis and fractionation of NAIP-harboring cells prior to affinity chromatography may be performed by standard methods (see e.g., Ausubel et al., *supra*). Once isolated, the recombinant protein can, if desired, be purified further by e.g., by high performance liquid chromatography (HPLC; e.g., see Fisher, Laboratory Techniques In Biochemistry And Molecular Biology, Work and Burdon, Eds., Elsevier, 1980).

Polypeptides of the invention, particularly short NAIP fragments, can also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL). These general techniques of polypeptide expression and purification can also be used to produce and isolate useful NAIP fragments or analogs, as described herein.

Those skilled in the art of molecular biology will understand that a wide variety of expression systems may be used to produce the recombinant protein. The precise host cell used is not critical to the invention. The NAIP protein may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *S. cerevisiae*, insect cells such as Sf21 cells, or mammalian cells such as COS-1, NIH 3T3, or HeLa cells). These cells are publically available, for example, from the American Type Culture Collection, Rockville, MD; see also Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1994). The method of transduction and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (*supra*), and expression vehicles may be

chosen from those provided, e.g. in Cloning Vectors: A Laboratory Manual (P.H. Pouwels et al., 1985, Supp. 1987).

III. Testing for the presence of NAIP biological activity

To analyze the effect of NAIP on apoptosis in a first approach, expression plasmids alone or encoding nearly full length NAIP or Bcl-2 (a protein which functions under normal conditions to protect cells against apoptosis) were transfected into CHO, Rat-1 and HeLa cells followed by G418 selection. Initially, a NAIP cDNA was isolated by probing a human fetal brain cDNA library with a genomic DNA insert of a cosmid from the constructed cosmid library, and a cDNA fragment encoding most of the three BIR domains corresponding to the NAIP gene sequence was isolated.

IV. Cellular Distribution of NAIP

We have looked at the distribution of NAIP using immunofluorescence of labelled antibodies and find NAIP is expressed in at least the following tissues: motor neurons, myocardial cells, liver, placenta and CNS.

V. NAIP Fragments

The BIR domains of NAIP appear to be both necessary and sufficient for NAIP biological activity. Surprisingly, we have reason to believe carboxy terminal deletions of NAIP amino acids actually enhances inhibition of apoptosis by NAIP. Deletions may be up to the end of the last NAIP BIR domain (i.e., the third), but need not delete the entire region carboxy terminal to the third BIR domains.

VI. NAIP Antibodies

In order to prepare polyclonal antibodies, NAIP, fragments of NAIP, or fusion proteins containing defined portions or all of the NAIP protein can be synthesized in bacteria by expression of corresponding DNA sequences in a suitable cloning vehicle. Fusion proteins are commonly used as a source of antigen for producing antibodies. Two widely used expression systems for *E.coli* are lacZ fusions using the pUR series of vectors and trpE fusions using the pATH vectors. The protein

can then be purified, coupled to a carrier protein and mixed with Freund's adjuvant (to help stimulate the antigenic response by the rabbits) and injected into rabbits or other laboratory animals. Alternatively, protein can be isolated from NAIP expressing cultured cells. Following booster injections at bi-weekly intervals, the rabbits or other laboratory animals are then bled and the sera isolated. The sera can be used directly or purified prior to use, by various methods including affinity chromatography employing Protein A-Sepharose, Antigen Sepharose, Anti-mouse-Ig-Sepharose. The sera can then be used to probe protein extracts from tissues run on a polyacrylamide gel to identify the NAIP protein. Alternatively, synthetic peptides can be made to the antigenic portions of the protein and used to innoculate the animals.

In order to generate peptide for use in making NAIP-specific antibodies, a NAIP coding sequence (i.e., amino acid fragments shown in Seq. ID Nos. 22 and 24) can be expressed as a C-terminal fusion with glutathione S-transferase (GST; Smith et al., Gene 67:31-40, 1988). The fusion protein can be purified on glutathione-Sepharose beads, eluted with glutathione, and cleaved with thrombin (at the engineered cleavage site), and purified to the degree required to successfully immunize rabbits. Primary immunizations can be carried out with Freund's complete adjuvant and subsequent immunizations performed with Freund's incomplete adjuvant. Antibody titres are monitored by Western blot and immunoprecipitation analyses using the thrombin-cleaved NAIP fragment of the GST-NAIP fusion protein. Immune sera are affinity purified using CNBr-Sepharose-coupled NAIP protein. Antiserum specificity is determined using a panel of unrelated GST proteins (including GSTp53, Rb, HPV-16 E6, and E6-AP) and GST-trypsin (which was generated by PCR using known sequences).

It is also understood by those skilled in the art that monoclonal NAIP antibodies may be produced by culturing cells actively expressing the protein or isolated from tissues. The cell extracts, or recombinant protein extracts, containing the NAIP protein, may for example, be injected in Freund's adjuvant into mice. After being injected, the mice spleens may be removed and resuspended in phosphate buffered saline (PBS). The spleen cells serve as a source of lymphocytes, some of which are producing antibody of the appropriate specificity. These are then fused with a permanently growing myeloma partner cells, and the products of the fusion are plated into a number

of tissue culture wells in the presence of a selective agent such as HAT. The wells are then screened by ELISA to identify those containing cells making binding antibody. These are then plated and after a period of growth, these wells are again screened to identify antibody-producing cells. Several cloning procedures are carried out until over 90% of the wells contain single clones which are positive for antibody production. From this procedure a stable line of clones which produce the antibody is established. The monoclonal antibody can then be purified by affinity chromatography using Protein A Sepharose, ion-exchange chromatography, as well as variations and combinations of these techniques. Truncated versions of monoclonal antibodies may also be produced by recombinant methods in which plasmids are generated which express the desired monoclonal antibody fragment(s) in a suitable host.

As an alternate or adjunct immunogen to GST fusion proteins, peptides corresponding to relatively unique hydrophilic regions of NAIP may be generated and coupled to keyhole limpet hemocyanin (KLH) through an introduced C-terminal lysine. Antiserum to each of these peptides is similarly affinity purified on peptides conjugated to BSA, and specificity is tested by ELISA and Western blotting using peptide conjugates, and by Western blotting and immunoprecipitation using NAIP expressed as a GST fusion protein.

Alternatively, monoclonal antibodies may be prepared using the NAIP proteins described above and standard hybridoma technology (see, e.g., Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, New York, NY, 1981; Ausubel et al., *supra*). Once produced, monoclonal antibodies are also tested for specific NAIP recognition by Western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., *supra*).

Antibodies that specifically recognize NAIP (or fragments of NAIP), such as those described herein containing one or more BIR domains are considered useful in the invention. They may, for example, be used in an immunoassay to monitor NAIP expression levels or to determine the subcellular location of a NAIP or NAIP fragment produced by a mammal. Antibodies that inhibit

NAIP described herein may be especially useful in inducing apoptosis in cells undergoing undesirable proliferation.

Preferably, antibodies of the invention are produced using NAIP sequence that does not reside within highly conserved regions, and that appears likely to be antigenic, as analyzed by criteria such as those provided by the Peptide structure program (Genetics Computer Group Sequence Analysis Package, Program Manual for the GCG Package, Version 7, 1991) using the algorithm of Jameson and Wolf (CABIOS 4:181, 1988). These fragments can be generated by standard techniques, e.g. by the PCR, and cloned into the pGEX expression vector (Ausubel et al., *supra*). Fusion proteins are expressed in *E. coli* and purified using a glutathione agarose affinity matrix as described in Ausubel et al. (*supra*). In order to minimize the potential for obtaining antisera that is non-specific, or exhibits low-affinity binding to NAIP, two or three fusions are generated for each protein, and each fusion is injected into at least two rabbits. Antisera are raised by injections in series, preferably including at least three booster injections.

VII. Use of NAIP Antibodies

Antibodies to NAIP may be used, as noted above, to detect NAIP or inhibit the protein. In addition, the antibodies coupled to compounds for diagnostic and/or therapeutic uses such as radionucleotides for imaging and therapy and liposomes for the targeting of compounds to a specific tissue location.

VIII. Detection of NAIP gene expression

As noted, the antibodies described above may be used to monitor NAIP protein expression. In addition, *in situ* hybridization is a method which may be used to detect the expression of the NAIP gene. *In situ* hybridization relies upon the hybridization of a specifically labelled nucleic acid probe to the cellular RNA in individual cells or tissues. Therefore, it allows the identification of mRNA within intact tissues, such as the brain. In this method, oligonucleotides or cloned nucleotide (RNA or DNA) fragments corresponding to unique portions of the NAIP gene are used to detect specific mRNA species, e.g., in the brain. In this method a rat is anesthetized and

transcardially perfused with cold PBS, followed by perfusion with a formaldehyde solution. The brain or other tissues is then removed, frozen in liquid nitrogen, and cut into thin micron sections. The sections are placed on slides and incubated in proteinase K. Following rinsing in DEP, water and ethanol, the slides are placed in prehybridization buffer. A radioactive probe corresponding to the primer is made by nick translation and incubated with the sectioned brain tissue. After incubation and air drying, the labelled areas are visualized by autoradiography. Dark spots on the tissue sample indicate hybridization of the probe with NAIP mRNA which demonstrates the expression of the protein.

IX. Identification of Molecules that Modulate NAIP Protein Expression

NAIP cDNAs may be used to facilitate the identification of molecules that increase or decrease NAIP expression. In one approach, candidate molecules are added, in varying concentration, to the culture medium of cells expressing NAIP mRNA. NAIP expression is then measured, for example, by Northern blot analysis (Ausubel et al., *supra*) using a NAIP cDNA, or cDNA or RNA fragment, as a hybridization probe. The level of NAIP expression in the presence of the candidate molecule is compared to the level of NAIP expression in the absence of the candidate molecule, all other factors (e.g. cell type and culture conditions) being equal.

The effect of candidate molecules on NAIP-mediated apoptosis may, instead, be measured at the level of translation by using the general approach described above with standard protein detection techniques, such as Western blotting or immunoprecipitation with a NAIP-specific antibody (for example, the NAIP antibody described herein).

Compounds that modulate the level of NAIP may be purified, or substantially purified, or may be one component of a mixture of compounds such as an extract or supernatant obtained from cells (Ausubel et al., *supra*). In an assay of a mixture of compounds, NAIP expression is tested against progressively smaller subsets of the compound pool (e.g., produced by standard purification techniques such as HPLC or FPLC) until a single compound or minimal number of effective compounds is demonstrated to modulate NAIP expression.

Compounds may also be screened for their ability to modulate NAIP apoptosis inhibiting activity. In this approach, the degree of apoptosis in the presence of a candidate compound is compared to the degree of apoptosis in its absence, under equivalent conditions. Again, the screen may begin with a pool of candidate compounds, from which one or more useful modulator compounds are isolated in a step-wise fashion. Apoptosis activity may be measured by any standard assay, for example, those described herein.

Another method for detecting compounds that modulate the activity of NAIPs is to screen for compounds that interact physically with a given NAIP polypeptide. These compounds may be detected by adapting interaction trap expression systems known in the art. These systems detect protein interactions using a transcriptional activation assay and are generally described by Gyuris et al. (Cell 75:791-803, 1993) and Field et al., Nature 340:245-246, 1989), and are commercially available from Clontech (Palo Alto, CA). In addition, PCT Publication WO 95/28497 describes an interaction trap assay in which proteins involved in apoptosis, by virtue of their interaction with Bcl-2, are detected. A similar method may be used to identify proteins and other compounds that interact with NAIP.

Compounds or molecules that function as modulators of NAIP-mediated cell death may include peptide and non-peptide molecules such as those present in cell extracts, mammalian serum, or growth medium in which mammalian cells have been cultured.

A molecule that promotes an increase in NAIP expression or NAIP activity is considered particularly useful in the invention; such a molecule may be used, for example, as a therapeutic to increase cellular levels of NAIP and thereby exploit the ability of NAIP polypeptides to inhibit apoptosis.

A molecule that decreases NAIP activity (e.g., by decreasing NAIP gene expression or polypeptide activity) may be used to decrease cellular proliferation. This would be advantageous in the treatment of neoplasms or other cell proliferative diseases.

Molecules that are found, by the methods described above, to effectively modulate NAIP gene expression or polypeptide activity may be tested further in animal models. If they continue to function successfully in an *in vivo* setting, they may be used as therapeutics to either inhibit or enhance apoptosis, as appropriate.

X. Therapies

Therapies may be designed to circumvent or overcome an NAIP gene defect or inadequate NAIP gene expression, and thus moderate and possibly prevent apoptosis. The NAIP gene is expressed in the liver, myocardium, and placenta, as well as in the CNS. Hence, in considering various therapies, it is understood that such therapies may be targeted at tissue other than the brain, such as the liver, myocardium, and any other tissues subsequently demonstrated to express NAIP.

a) Protein Therapy

Treatment or prevention of apoptosis can be accomplished by replacing mutant or insufficient NAIP protein with normal protein, by modulating the function of mutant protein, or by delivering normal NAIP protein to the appropriate cells. Once the biological pathway of the NAIP protein has been completely understood, it may also be possible to modify the pathophysiologic pathway (e.g., a signal transduction pathway) in which the protein participates in order to correct the physiological defect.

To replace a mutant protein with normal protein, or to add protein to cells which no longer express sufficient NAIP, it is necessary to obtain large amounts of pure NAIP from cultured cell systems which can express the protein. Delivery of the protein to the affected tissues can then be accomplished using appropriate packaging or administrating systems. Alternatively, small molecule analogs may be used and administered to act as NAIP agonists and in this manner produce a desired physiological effect. Methods for finding such molecules are provided herein.

b) Gene Therapy

Gene therapy is another potential therapeutic approach in which normal copies of the NAIP gene are introduced into selected tissues to successfully code for normal and abundant protein in

affected cell types. The gene must be delivered to those cells in a form in which it can be taken up and code for sufficient protein to provide effective function. Alternatively, in some mutants it may be possible to prevent apoptosis by introducing another copy of the homologous gene bearing a second mutation in that gene or to alter the mutation, or use another gene to block any negative effect.

Transducing retroviral vectors can be used for somatic cell gene therapy especially because of their high efficiency of infection and stable integration and expression. The targeted cells however must be able to divide and the expression of the levels of normal protein should be high. The full length NAIP gene, or portions thereof, can be cloned into a retroviral vector and driven from its endogenous promoter or from the retroviral long terminal repeat or from a promoter specific for the target cell type of interest (such as neurons). Other viral vectors which can be used include adeno-associated virus, vaccinia virus, bovine papilloma virus, or a herpes virus such as Epstein-Barr virus.

Gene transfer could also be achieved using non-viral means requiring infection *in vitro*. This would include calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes may also be potentially beneficial for delivery of DNA into a cell. Although these methods are available, many of these are lower efficiency.

Antisense based strategies can be employed to explore NAIP gene function and as a basis for therapeutic drug design. The principle is based on the hypothesis that sequence-specific suppression of gene expression can be achieved by intracellular hybridization between mRNA and a complementary antisense species. The formation of a hybrid RNA duplex may then interfere with the processing/transport/translation and/or stability of the target NAIP mRNA. Antisense strategies may use a variety of approaches including the use of antisense oligonucleotides, injection of antisense RNA and transfection of antisense RNA expression vectors. Antisense effects can be induced by control (sense) sequences, however, the extent of phenotypic changes are highly variable. Phenotypic effects induced by antisense effects are based on changes in criteria such as protein levels, protein activity measurement, and target mRNA levels.

Transplantation of normal genes into the affected cells of a patient can also be useful therapy. In this procedure, normal NAIP is transferred into a cultivatable cell type , either exogenously or endogenously to the patient. These cells are then injected serotologically into the targeted tissue(s).

Retroviral vectors, adenoviral vectors, adeno associated viral vectors, or other viral vectors with the appropriate tropism for cells likely to be involved in apoptosis (for example, epithelial cells) may be used as a gene transfer delivery system for a therapeutic NAIP gene construct. Numerous vectors useful for this purpose are generally known (Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis and Anderson, BioTechniques 6:608-614, 1988; Tolstoshev and Anderson, current opinion in Biotechnology 1:55-61, 1990; Sharp, The Lancet 337:1277-1278, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, Blood Cells 17:407-416, 1991; Miller et al., Biotechniques 7:980-990, 1989; Le Gal La Salle et al., Science 259:988-990, 1993; and Johnson, Chest 107:77S-83S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med 323:370, 1990; Anderson et al., U.S. Patent No. 5,399,346). Non-viral approaches may also be employed for the introduction of therapeutic DNA into cells otherwise predicted to undergo apoptosis. For example, NAIP may be introduced into a neuron or a T cell by lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413, 1987; Ono et al., Neurosci. Lett. 117:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Meth. Enz. 101:512, 1983), asialorosonucoid-polylysine conjugation (Wu et al., J. Biol. Chem. 263:14621, 1988; Wu et al., J. Biol. Chem. 264:16985, 1989); or, less preferably, microinjection under surgical conditions (Wolff et al., Science 247:1465, 1990).

For any of the methods of application described above, the therapeutic NAIP DNA construct is preferably applied to the site of the predicted apoptosis event (for example, by injection). However, it may also be applied to tissue in the vicinity of the predicted apoptosis event or to a blood vessel supplying the cells predicted to undergo apoptosis.

In the constructs described, NAIP cDNA expression can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in neural cells, T cells, or B cells may be used to direct NAIP expression. The enhancers used could include, without limitation, those that are characterized as tissue- or cell-specific in their expression. Alternatively, if a NAIP genomic clone is used as a therapeutic construct (for example, following its isolation by hybridization with the NAIP cDNA described above), regulation may be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

Less preferably, NAIP gene therapy is accomplished by direct administration of the NAIP mRNA or antisense NAIP mRNA to a cell that is expected to undergo apoptosis. The mRNA may be produced and isolated by any standard technique, but is most readily produced by *in vitro* transcription using a NAIP cDNA under the control of a high efficiency promoter (e.g., the T7 promoter). Administration of NAIP antisense or mRNA to cells mRNA can be carried out by any of the methods for direct nucleic acid administration described above.

Ideally, the production of NAIP protein by any gene therapy approach will result in cellular levels of NAIP that are at least equivalent to the normal, cellular level of NAIP in an unaffected cell. Treatment by any NAIP-mediated gene therapy approach may be combined with more traditional therapies.

Another therapeutic approach within the invention involves administration of recombinant NAIP protein, either directly to the site of a predicted apoptosis event (for example, by injection) or systemically (for example, by any conventional recombinant protein administration technique). The dosage of NAIP depends on a number of factors, including the size and health of the individual patient, but, generally, between **[O.l mg and 100 mg]** inclusive are administered per day to an adult in any pharmaceutically acceptable formulation.

XI. Administration of NAIP Polypeptides, NAIP Genes, or Modulators of NAIP Synthesis or Function

A NAIP protein, gene, or modulator may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer NAIP to patients suffering from a disease that is caused by excessive apoptosis. Administration may begin before the patient is symptomatic. Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intraarterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for NAIP modulatory compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

If desired, treatment with a NAIP protein, gene, or modulatory compound may be combined with more traditional therapies for the disease such as surgery, steroid therapy, or chemotherapy for

autoimmune disease; antiviral therapy for AIDS; and tissue plasminogen activator (TPA) for ischemic injury.

XII. Detection of Conditions Involving Altered Apoptosis

NAIP polypeptides and nucleic acid sequences find diagnostic use in the detection or monitoring of conditions involving aberrant levels of apoptosis. For example, decrease expression of NAIP may be correlated with enhanced apoptosis in humans (see XII, below). Accordingly, a decrease or increase in the level of NAIP production may provide an indication of a deleterious condition. Levels of NAIP expression may be assayed by any standard technique. For example, NAIP expression in a biological sample (e.g., a biopsy) may be monitored by standard Northern blot analysis or may be aided by PCR (see, e.g., Ausubel et al., *supra*; PCR Technology: Principles and Applications for DNA Amplification, H.A. Ehrlich, Ed. Stockton Press, NY; Yap et al. Nucl. Acids. Res. 19:4294, 1991).

Alternatively, a biological sample obtained from a patient may be analyzed for one or more mutations in the NAIP sequences using a mismatch detection approach. Generally, these techniques involve PCR amplification of nucleic acid from the patient sample, followed by identification of the mutation (i.e., mismatch) by either altered hybridization, aberrant electrophoretic gel migration, binding or cleavage mediated by mismatch binding proteins, or direct nucleic acid sequencing. Any of these techniques may be used to facilitate mutant NAIP detection, and each is well known in the art; examples of particular techniques are described, without limitation, in Orita et al., Proc. Natl. Acad. Sci. USA 86:2766-2770, 1989; Sheffield et al., Proc. Natl. Acad. Sci. USA 86:232-236, 1989).

In yet another approach, immunoassays are used to detect or monitor NAIP protein in a biological sample. NAIP specific polyclonal or monoclonal antibodies (produced as described above) may be used in any standard immunoassay format (e.g., ELISA, Western blot, or RIA) to measure NAIP polypeptide levels. These levels would be compared to wild-type NAIP levels, with a decrease in NAIP production indicating a condition involving increased apoptosis. Examples of immunoassays are described, e.g., in Ausubel et al., *supra*. Immunohistochemical techniques may

also be utilized for NAIP detection. For example, a tissue sample may be obtained from a patient, sectioned, and stained for the presence of NAIP using an anti-NAIP antibody and any standard detection system (e.g., one which includes a secondary antibody conjugated to horseradish peroxidase). General guidance regarding such techniques can be found in, e.g., Bancroft and Stevens (Theory and Practice of Histological Techniques, Churchill Livingstone, 1982) and Ausubel et al. (*supra*).

In one preferred example, a combined diagnostic method may be employed that begins with an evaluation of NAIP protein production (for example, by immunological techniques or the protein truncation test (Hogerrorst et al., Nature Genetics 10:208-212, 1995) and also includes a nucleic acid-based detection technique designed to identify more subtle NAIP mutations (for example, point mutations). As described above, a number of mismatch detection assays are available to those skilled in the art, and any preferred technique may be used. Mutations in NAIP may be detected that either result in loss of NAIP expression or loss of NAIP biological activity. In a variation of this combined diagnostic method, NAIP biological activity is measured as anti-apoptotic activity using any appropriate apoptosis assay system (for example, those described herein).

Mismatch detection assays also provide an opportunity to diagnose a NAIP-mediated predisposition to diseases caused by inappropriate apoptosis. For example, a patient heterozygous for a NAIP mutation may show no clinical symptoms and yet possess a higher than normal probability of developing one or more types of neurodegenerative, myelodysplastic or having severe sequelae to an ischemic event. Given this diagnosis, a patient may take precautions to minimize their exposure to adverse environmental factors (for example, UV exposure or chemical mutagens) and to carefully monitor their medical condition (for example, through frequent physical examinations). This type of NAIP diagnostic approach may also be used to detect NAIP mutations in prenatal screens. The NAIP diagnostic assays described above may be carried out using any biological sample (for example, any biopsy sample or other tissue) in which NAIP is normally expressed. Identification of a mutant NAIP gene may also be assayed using these sources for test samples.

Alternatively, a NAIP mutation, particularly as part of a diagnosis for predisposition to NAIP-associated degenerative disease, may be tested using a DNA sample from any cell, for example, by mismatch detection techniques. Preferably, the DNA sample is subjected to PCR amplification prior to analysis.

XIII. Preventative Anti-Apoptotic Therapy

In a patient diagnosed to be heterozygous for a NAIP mutation or to be susceptible to NAIP mutations (even if those mutations do not yet result in alteration or loss of NAIP biological activity), or a patient diagnosed with a degenerative disease (e.g., motor neuron degenerative diseases such as SMA or ALS diseases), or diagnosed as HIV positive, any of the above therapies may be administered before the occurrence of the disease phenotype. For example, the therapies may be provided to a patient who is HIV positive but does not yet show a diminished T cell count or other overt signs of AIDS. In particular, compounds shown to increase NAIP expression or NAIP biological activity may be administered by any standard dosage and route of administration (see above). Alternatively, gene therapy using a NAIP expression construct may be undertaken to reverse or prevent the cell defect prior to the development of the degenerative disease.

The methods of the instant invention may be used to reduce or diagnose the disorders described herein in any mammal, for example, humans, domestic pets, or livestock. Where a non-human mammal is treated or diagnosed, the NAIP polypeptide, nucleic acid, or antibody employed is preferably specific for that species.

XV. Identification of Additional NAIP Genes

Standard techniques, such as the polymerase chain reaction (PCR) and DNA hybridization, may be used to clone additional NAIP homologues in other species. Southern blots of murine genomic DNA hybridized at low stringency with probes specific for human NAIP reveal bands that correspond to NAIP and/or related family members. Thus, additional NAIP sequences may be readily identified using low stringency hybridization. Examples of murine and human NAIP-specific primers, which may be used to clone additional genes by RT-PCR.

XVI. Characterization of NAIP Activity and Intracellular Localization Studies

The ability of NAIP to modulate apoptosis can be defined in *in vitro* systems in which alterations of apoptosis can be detected. Mammalian expression constructs carrying NAIP cDNAs, which are either full-length or truncated, can be introduced into cell lines such as CHO, NIH 3T3, HL60, Rat-1, or Jurkat cells. In addition, SF21 insect cells may be used, in which case the NAIP gene is preferentially expressed using an insect heat shock promotor. Following transfection, apoptosis can be induced by standard methods, which include serum withdrawal, or application of staurosporine, menadione (which induces apoptosis via free radical formation), or anti-Fas antibodies. As a control, cells are cultured under the same conditions as those induced to undergo apoptosis, but either not transfected, or transfected with a vector that lacks a NAIP insert. The ability of each NAIP construct to inhibit apoptosis upon expression can be quantified by calculating the survival index of the cells, i.e., the ratio of surviving transfected cells to surviving control cells. These experiments can confirm the presence of apoptosis inhibiting activity and, as discussed below, can also be used to determine the functional region(s) of a NAIP. These assays may also be performed in combination with the application of additional compounds in order to identify compounds that modulate apoptosis via NAIP expression.

XVII. Examples of Additional Apoptosis Assays

Specific examples of apoptosis assays are also provided in the following references. Assays for apoptosis in lymphocytes are disclosed by: Li et al., "Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein", Science 268:429-431, 1995; Gibellini et al., "Tat-expressing Jurkat cells show an increased resistance to different apoptotic stimuli, including acute human immunodeficiency virus-type 1 (HIV-1) infection", Br. J. Haematol. 89:24-33, 1995; Martin et al., "HIV-1 infection of human $CD4^+$ T cells *in vitro*. Differential induction of apoptosis in these cells." J. Immunol. 152:330-42, 1994; Terai et al., "Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1", J. Clin Invest. 87:1710-5, 1991; Dhein et al., "Autocrine T-cell suicide mediated by APO-1/(Fas/CD95)11, Nature 373:438-441, 1995; Katsikis et al., "Fas antigen stimulation induces marked apoptosis of T lymphocytes in human

immunodeficiency virus-infected individuals", J. Exp. Med. 1815:2029-2036, 1995; Westendorp et al., Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp12O", Nature 375:497, 1995; DeRossi et al., Virology 198:234-44, 1994.

Assays for apoptosis in fibroblasts are disclosed by: Vossbeck et al., "Direct transforming activity of TGF-beta on rat fibroblasts", Int. J. Cancer 61:92-97, 1995; Goruppi et al., "Dissection of c-myc domains involved in S phase induction of NIH3T3 fibroblasts", Oncogene 9:1537-44, 1994; Fernandez et al., "Differential sensitivity of normal and Ha-ras transformed C3H mouse embryo fibroblasts to tumor necrosis factor: induction of bcl-2, c-myc, and manganese superoxide dismutase in resistant cells", Oncogene 9:2009-17, 1994; Harrington et al., "c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines", EMBO J., 13:3286-3295, 1994; Itoh et al., "A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen", J. Biol. Chem. 268:10932-7, 1993.

Assays for apoptosis in neuronal cells are disclosed by: Melino et al., "Tissue transglutaminase and apoptosis: sense and antisense transfection studies with human neuroblastoma cells", Mol. Cell Biol. 14:6584-6596, 1994; Rosenbaum et al., "Evidence for hypoxia-induced, programmed cell death of cultured neurons", Ann. Neurol. 36:864-870, 1994; Sato et al., "Neuronal differentiation of PC12 cells as a result of prevention of cell death by bcl-2", J. Neurobiol 25:1227-1234, 1994; Ferrari et al., "N-acetylcysteine D- and L-stereoisomers prevents apoptotic death of neuronal cells", J. Neurosci. 1516:2857-2866, 1995; Talley et al., "Tumor necrosis factor alpha-induced apoptosis in human neuronal cells: protection by the antioxidant N-acetylcysteine and the genes bcl-2 and crma", Mol. Cell Biol. 1585:2359-2366, 1995; Talley et al., "Tumor Necrosis Factor Alpha-Induced Apoptosis in Human Neuronal Cells: Protection by the Antioxidant N-Acetylcysteine and the Genes bcl-2 and crma", Mol. Cell. Biol. 15:2359-2366, 1995; Walkinshaw et al., "Induction of apoptosis in catecholaminergic PC12 cells by L-DOPA. Implications for the treatment of Parkinson's disease.", J. Clin. Invest. 95:2458-2464, 1995.

Assays for apoptosis in insect cells are disclosed by: Clem et al., "Prevention of apoptosis by a baculovirus gene during infection of insect cells", Science 254:1388-90, 1991; Crook et al.,

"An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif", J. Virol. 67:2168-74, 1993; Rabizadeh et al., "Expression of the baculovirus p35 gene inhibits mammalian neural cell death", J. Neurochem. 61:2318-21, 1993; Birnbaum et al., "An apoptosis inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs", J. Virol. 68:2521-8, 1994; Clem et al., *Mol. Cell. Biol.* 14:5212-5222, 1994.

XVIII. Construction of a Transgenic Animal

Characterization of NAIP genes provides information that is necessary for a NAIP knockout animal model to be developed by homologous recombination. Preferably, the model is a mammalian animal, most preferably a mouse. Similarly, an animal model of NAIP overproduction may be generated by integrating one or more NAIP sequences into the genome, according to standard transgenic techniques.

A replacement-type targeting vector, which would be used to create a knockout model, can be constructed using an isogenic genomic clone, for example, from a mouse strain such as 129/Sv (Stratagene Inc., LaJolla, CA). The targeting vector will be introduced into a suitably-derived line of embryonic stem (ES) cells by electroporation to generate ES cell lines that carry a profoundly truncated form of a NAIP. To generate chimeric founder mice, the targeted cell lines will be injected into a mouse blastula stage embryo. Heterozygous offspring will be interbred to homozygosity. Knockout mice would provide the means, *in vivo*, to screen for therapeutic compounds that modulate apoptosis via an NAIP-dependent pathway. Making such mice may require use of loxP sites due to the multiple copies of NAIP on the chromosome (see Sauer and Henderson, Nucleic Aids Res. 17: 147-61 (1989)).

Examples

The examples are meant to illustrate, not limit the invention.

Example 1 Expression of NAIP in Rat-1, CHO and HeLa pooled stable lines and adenovirus infected cells analysed by Western blotting and immunofluorescence.

To generate nearly 3.7 kb NAIP construct tagged with the myc epitope (I) MTG-SP3.7, a 2.5 kb Bsu36I/SalI fragment of NAIP cloned into Bluescript and (ii) Bsu36I/XhoI cut MTG-SE1.7, the expression vector pcDNA3 containing a 300 bp myc epitope and a 1.7 kb fragment of NAIP were ligated. HeLa, CHO and Rat-1 cells were transfected by lipofection (Gibco BRL) with 8 μg DNA and G418 resistant transformants were selected by maintaining the cells in 250 μg/ml, 400 μg/ml and 800μg/ml G418 respectively. All cells were maintained in Eagles medium containing 10% fetal calf serum. For construction of the adenovirus, a 3.7 kb BamHI fragment of NAIP was cloned into the SwaI site of the adenovirus expression cosmid pAdex1CAwt. Production of vectors, purification by double cesium chloride gradient and titer determination was as described in Rosenfeld, M.A. *et. al.* 1992, and Graham, F.L. and Van Der Eb, A. 1973.

Western blot analysis was performed using mouse anti-human myc monoclonal antibody (Ellison, M.J. and Hochstrasser, M.J. 1991) or rabbit anti-human NAIP (E1.0) polyclonal antibody. For NAIP antibody production, rabbits were immunized with purified bacterial produced fusion protein in complete Freunds adjuvant. Serum was pre-cleared with GST protein and anti-NAIP immunoglobin purified with immobilized GST-NAIP fusion proteins.

For immunofluorescence, cells were grown on glass slides, fixed with formaldehyde for 10 minutes, incubated with anti-NAIP (1:200) or anti-myc (1:20) in PBS, 0.3% Triton X-100™ for 1 hour followed by incubation with secondary antisera, FITC-labelled donkey anti-rabbit immunoglobulin (Amersham), biotinylated goat anti-mouse immunoglobulin (Amersham) and streptavidin Texas-Red™ (Amersham).

Example 2 The Effect of NAIP on Cell Death Induced by Serum Deprivation, Menadione and TNF-α.

For each assay cells were plated at 5 x 104 ml in triplicate. CHO or Rat-1 cells were treated with menadione for 1.5 hours, washed 5 times in PBS and maintained in normal media. For serum deprivation assays, cells were washed 5 times in PBS and maintained in media with 0% fetal calf serum. HeLa cells were treated with 20 units/ml TNF-α in combination with 30 g/ml cyclohexamide for 17 hours. Apoptosis was assayed for each trigger by propidium iodide staining.

Adenovirus infected cells were subjected to triggers 36 hours post infection. LacZ expression was confirmed histochemically by 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) as described in Ellison, M.J. and Hochstrasser, M.J. 1991. Transcription of PIAN was determined by *in situ* hybridization using the DIG labelled sense oligonucleotide following the manufacturers protocol (Boehringer Mannheim). The human Bcl-2 clone pB4 (ATCC) was digested with EcoRI and ligated into the EcoRI site of pcDNA3.

For adenovirus assays an adenovirus encoding LacZ, antisense NAIP (NAIP) or vector alone with no insert were utilized as controls. Bcl-2 was utilized as a positive control and pcDNA alone as a negative control in cell line assays. Cell viability was determined by trypan blue exclusion. Date are presented as averages of three independently derived transfected pools or infections.

Example 3 Immunofluorescence Analysis of Human Spinal Cord Tissue.

Human tissues were obtained at autopsy from a 2 month old infant that died of non-neurological causes and stored at -80°C. 14 μM cryostat sections were fixed in formaldehyde for 20 minutes, rinsed in PBS and incubated in blocking solution (2% horse serum, 2% casien, 2% BSA in PBS) for 15 minutes prior to overnight incubation with anti-NAIP antisera diluted in this blocking solution. CY-3 labelled donkey anti-rabbit immunoglobulin (Sigma) was utilized as secondary antisera.

Example 4 Isolating and cloning the NAIP gene

PAC Contig Array

The 40G1 CATT subloci demonstrated linkage disequilibrium and therefore a PAC contiguous array containing the CATT region was constructed. This PAC contig array comprised 9 clones and extended approximately 400 kb. Genetic analysis combined with the physical mapping data indicated that the 40G1 CATT subloci marker which showed the greatest disequilibrium with SMA was duplicated and was localized at the extreme centromeric of the critical SMA interval. Consequently the 154 kb PAC clone 125D9 which contained within 10 kb of its centromeric end the

SMA interval defining CMS allele 9 and extended telemetrically to incorporate the 40G1 CATT sublocus was chosen for further examination.

Two genomic libraries were constructed by performing complete and partial (average insert size 5 kb) Sau3A1 on PAC 125D9 and cloning the restricted products into BamH1 digested Bluescript plasmids. Genomic sequencing was conducted on both termini of 200 clones from the 5 kb insert partial Sau3A1 library in the manner of (Chen et al., 1993) permitting the construction of contiguous and overlapping genomic clones covering most of the PAC. This proved instrumental in the elucidation of the neuronal apoptosis inhibitor protein gene structure.

PAC 125D9 is cleaved into 30 kb centromeric and 125 kb telomeric fragments by a NotI site (which was later shown to bisect exon 7 of the PAC 125D9 at the beginning of the apoptosis inhibitor domain. The NotI PAC fragments were isolated by preparative PFGE and used separately to probe fetal brain cDNA libraries. Physical mapping and sequencing of the NotI site region was also undertaken to assay for the presence of a CpG island, an approach which rapidly detected coding sequences. The PAC 125D9 was also used as a template in an exon trapping system resulting in the identification of the exons contained in the neuronal apoptosis inhibitor protein gene.

The multipronged approach, in addition to the presence of transcripts identified previously by hybridization by clones from the cosmid array (such as, GA1 and L7), resulted in the rapid identification of six cDNA clones contained in neuronal apoptosis inhibitor protein gene. The clones were arranged, where possible, into overlapping arrays. Chimerism was excluded on a number of occasions by detection of co-linearity of the cDNA clone termini with sequences from clones derived from the PAC 125D9 partial Sau3A1 genomic library.

Cloning of Neuronal Apoptosis Inhibitor Protein Gene

A human fetal spinal cord cDNA library was probed with the entire genomic DNA insert of cosmid 250B6 containing one of the 5 CATT subloci. This resulted in a detection of a 2.2 kb transcript referred to as GA1. Further probings of fetal brain libraries with the contiguous cosmid

inserts (cosmids 40G1) as well as single copy subclones isolated from such cosmids were undertaken. A number of transcripts were obtained including one termed L7. No coding region was detected for L7 probably due to the fact that a substantial portion of the clone contained unprocessed heteronuclear RNA. However, it was later discovered that L7 proved to comprise part of what is believed to be the neuronal apoptosis inhibitor protein gene. Similarly, the GA1 transcript ultimately proved to be exon 13 of the neuronal apoptosis inhibitor protein. Since GA1 was found to contain exons indicating that it was an expressed gene, it was of particular interest. The GA1 transcript which was contained within the PAC clone 125D9 was subsequently extended by further probing in cDNA libraries.

The remaining gaps in the cDNA were completed and the final 3' extension was achieved by probing a fetal brain library with two trapped exons. A physical map of the cDNA with overlapping clones was prepared. The entire cDNA sequence is shown in Table 1 and contains 18 exons (1 to 14a and 14 to 17). The amino acid sequence starts with methionine which corresponds to the nucleotide triplet ATG.

DNA Manipulation and Analysis

Four genomic libraries containing PAC 125D9 insert were constructed by BamHI, BamHI/NotI, total and partial Sau3aI (selected for 5kb insert size) digestions of the PAC genomic DNA insert and subcloned into Bluescript vector. Sequencing of approximately 400 bp of both termini of 200 five kb clones from the partial Sau3AI digestion library in the manner of Chen et al. (1993) was undertaken.

Coding sequences from the PACs were isolated by the exon amplification procedure as described by Church et al. (1994). PACs were digested with BamHI or BamHI and BglII and subcloned into pSPL3. Pooled clones of each PAC were transfected into COS-1 cells. After a 24h transfection total RNA was extracted. Exons were cloned into pAMP10 (Gibco, BRL) and sequenced utilizing primer SD2 (GTG AAC TGC ACT GTG ACA AGC TGC).

DNA sequencing was conducted on an ABI 373A automated DNA sequencer. Two commercial human fetal brain cDNA libraries in lambda gt (Stratagene) and lambda ZAP (Clontech) were used for candidate transcript isolation. The Northern blot was commercially acquired (Clontech) and probing was performed using standard methodology.

In general, primers used in the paper for PCR were selected for T_ms of 60°C and can be used with the following conditions: 30 cycles of 94°C, 60s; 60°C, 60s; 72°C, 90s. PCR primer mappings are as referred to in the figure legends and text. Primer sequences are as follows:

1258 ATg CTT ggA TCT CTA gAA Tgg - Sequence ID No. 3

1285 AgC AAA gAC ATg Tgg Cgg AA - Sequence ID No. 4

1343 CCA gCT CCT AgA gAA AgA Agg A - Sequence ID No. 5

1844 gAA CTA Cgg CTg gAC TCT TTT - Sequence ID No. 6

1863 CTC TCA gCC TgC TCT TCA gAT - Sequence ID No. 7

1864 AAA gCC TCT gAC gAg Agg ATC - Sequence ID No. 8

1884 CgA CTg CCT gTT CAT CTA CgA - Sequence ID No. 9

1886 TTT gTT CTC CAg CCA CAT ACT - Sequence ID No. 10

1887 CAT TTg gCA TgT TCC TTC CAA g - Sequence ID No. 11

1893 gTA gAT gAA TAC TgA TgT TTC ATA ATT - Sequence ID No. 12

1910 TgC CAC TgC CAg gCA ATC TAA - Sequence ID No. 13

1919 TAA ACA ggA CAC ggT ACA gTg - Sequence ID No. 14

1923 CAT gTT TTA AgT CTC ggT gCT CTg - Sequence ID No. 15

1926 TTA gCC AgA TgT gTT ggC ACA Tg - Sequence ID No. 16

1927 gAT TCT ATg TgA TAg gCA gCC A - Sequence ID No. 17

1933 gCC ACT gCT CCC gAT ggA TTA - Sequence ID No. 18

1974 gCT CTC AgC TgC TCA TTC AgA T - Sequence ID No. 19

1979 ACA AAg TTC ACC ACg gCT CTg - Sequence ID No. 20

Our genetic and mapping analysis of SMA has led to the identification of the 154 kb insert of PAC125D9 as the likely site of the SMA gene. We report here the complete DNA sequence of the 131 kb portion of the PAC125D9 insert which contains both NAIP and SMN[tel] as well as the 3' end of a copy of the Basic Transcription Factor gene BTF2p44.[9] PAC125D9 insert digested with a variety of restriction enzymes was used to generate nine libraries. Shotgun sequencing of clones from the Sau3A1 library was hampered by the Alu rich nature of the area, sequencing was therefore conducted by a modified transposon based approach[10] yielding the configuration depicted in the figure. The NAIP and SMN[tel] genes, separated by 15.5 kb, are in a tail to tail (5'-->3':3'<--5') orientation, spanning 56 kb and 28 kb of genomic DNA, respectively. The gene BTF2p44 exists in a number of copies on 5q13.1[10]; exons 11-16 of one BTF2P44 copy occupy the most 5' eleven kb of the PAC insert followed by an 11 kb interval before NAIP exon 2. The first NAIP exon as originally reported[3] is not present in this PAC and may have been a heteronuclear artifact. An approximately 3 kb section of the 15.5 kb interval between NAIP and SMN (CCA, figure) is transcribed but contains no protein coding sequence. Indeed, no coding sequence in addition to BTF2P44, NAIP and SMN was identified throughout the entire interval.

CpG islands were identified in the 5' region of both SMN and NAIP genes. One hundred and forty five Alu sequences were identified in the 131 kb sequence, with five clusters of high density seen (figure legend). Such Alu density associated with L1 paucity (five copies) is in keeping with previous findings for light Giemsa staining (or reverse) chromosomal bands[11]. Copies of other repeats (e.g. MIR2, MST and MER) as detected by Sequin program are also as depicted[12]. The polymorphic microsatellite loci previously mapped to the SMA region; (CMS1[13], CATT[14] or C161[15], C171[15], C272[15] or AG-1[16,17]) as well as unusual single and di-nucleotide repeats are as shown.

The full length NAIP cDNA (6228 bp with an ORF of 4212 bp) was also elucidated by cDNA sequencing and comparison with PAC sequence, comprising 17 exons encoding a predicted 156 kDa protein of 1403 amino acids (data not shown). A novel NAIP exon 14 between the original exon 14 and 15 was identified. The original exon 17 has been replaced by a novel exon which

contains the stop codon, a 1.6 kb 3' UTR region and the polyadenylation consensus site (AATAAA) identified by 3' RACE. No new protein domains are found in the NAIP gene.

A rigorous definition of how far deletions extend on type 1 SMA chromosomes is central to our understanding of disease pathogenesis. If the genotype most frequently observed on type 1 SMA chromosomes (i.e. absence of NAIP exons 4 and 5 as well as SMN^{tel} exons 7 and 8) are the result of a single event, then our sequencing suggests a minimal deletion size of 60 kb. The high deletion frequency on type 1 SMA chromosomes of the CATT-40G1[14], (which maps between NAIP exon 7 and 8) is consistent with such a deletion.

Southern blots containing genomic DNA probed with NAIP cDNA reveal a diversity of bands, a result of the polymorphic number of variant forms of this locus mapping to 5q13.1[3,18]. In contrast, the same blots probed with SMN cDNA reveals only the bands associated with the intact SMN locus, for SMA and non-SMA individuals alike. Thus, there is no evidence of truncated or partially deleted SMN genes such as seen with the NAIP gene. The absence of any detectable SMN junction fragment in SMA patients strongly suggests that the SMN^{tel} exon 7 and 8 deletion detected in the significant majority of SMA cases incorporates the entire SMN^{tel} gene, thus extending the putative minimal SMA type 1 deletion to approximately 100 kb (figure). This is in keeping with the high deletion frequency of C272[15] (or AG-1[16,17]) microsatellite (which maps to SMN exon 1, figure) on type 1 SMA chromosomes. A 15% deletion frequency of one copy of BTF2P44 is observed in all SMA cases irrespective of clinical severity[9], suggesting that this mutation may not be an extension of the putative SMN-NAIP deletion. Clarification of this issue must await details of which copy of p44 is deleted.

Our sequencing of PAC125D9 maps the intact NAIP locus and clinically relevant SMN^{tel} to a 100 kb region which contains those microsatellite polymorphisms that are preferentially deleted on the significant majority of type 1 SMA chromosomes (i.e. CATT-40G1[14] C272[15] or AG-1[16,17]). The absence of any protein coding sequence, other than NAIP and SMN in this interval, focuses attention on these two genes as the key modulators of type 1 SMA. One potential pathogenic model is that SMN^{tel} absence acts as the primary neurotoxic insult[19] with NAIP depletion/absence leading

to an attenuated apoptotic resistance[5,6], exacerbating motor neuron attrition. Presence of additional SMN^{cen} may also act to modulate the course of the disease[20]. In addition to aiding in our comprehension of the molecular pathology of acute SMA, the sequence presented here should help in the study of transcriptional control elements for both genes, possibly facilitating the formulation of genetic therapies for this devastating neuromuscular disease.

DNA Sequencing

Partial Sau3A1 (selected for 3-5kb) BamHI, EcoRI, HindIII, PstI, SstI, XbaI and EcoRV libraries) were made from the PAC125D9 insert and sequenced using a transposon-based methodology (TN1000 Gold Biotechnology[10]). Subcloning of a large number of inserts into the commercially supplied pMOB plasmid was found to be problematic, therefore pUC18 and pBluescript SK were used. In general, fewer than 10% of clones had transposons in the vector region. *E. coli* lysate was employed as sequencing template using our modified heat soaked protocol[21]. Sequencing was from the TN1000 transposon randomly inserted into the target DNA, using primers of opposite orientation (5'-ATA TAA ACA ACGAAT TAT CTC C-3'; 5'-GTA TTA TAA TCA ATA AGTTAT ACC-3'), generating approximately 1 kb of sequence with a 5 bp overlap, easily spanning 300bp Alu repeats. Our approach permitted sequencing of inserts as large as 14 kb.

As the SMA region is known to be unstable, special care to ensure an intact, unaltered PAC insert was undertaken primarily by comparison of PAC125D9 insert and genomic DNA hybridization patterns on Southern blots.

Raw DNA sequence data generated by our automated sequencers (ABI 373 and ABI 373A) were processed and assembled in parallel by the Sequencher 3.0 program (Gene Codes Inc.); and the GAP4 program from the Staden package[27]. The edited results were automatically converted into GCG file formats[22] and placed in a separate database for searches by outside users using our e-mail server at smafasta@mgcheo.med.uottawa.ca. GRAIL[28] and Blast[29] searches were employed to screen for protein coding sequence and the PROSITE Protein database[24] was used to search for protein domains.

Example 5 NAIP Expression Vectors

Using the identified NAIP sequence information, a full length 3.7 kb NAIP construct tagged with the myc epitope (I) MTG-SP3.7, a 2.5 kb Bsu36I/SalI fragment of NAIP cloned into Bluescript and (ii) Bsu36I/XhoI cut MTG-SE1.7, the expression vector pcDNA3 containing a 300 bp myc epitope and a 1.7 kb fragment of NAIP were ligated. HeLa, CHO and Rat-1 cells were transfected by lipofection (Gibco BRL) with 8 μg DNA and G418 resistant transformants were selected by maintaining the cells in 250 μg/ml, 400 μg/ml and 800μg/ml G418 respectively.

In a second approach, cells were infected with adenovirus alone or adenovirus expressing either NAIP, antisense NAIP, or LacZ. For construction of the adenovirus, a 3.7 kb BamHI fragment of NAIP was cloned into the SwaI site of the adenovirus expression cosmid pAdex1CAwt. The antisense NAIP RNA contains a sequence complementary to the region of an mRNA containing an initiator codon. Expression of NAIP was confirmed in both procedures by Western blot analysis and immunofluorescence. Following infection with the recombinant adenoviruses, CHO cells were induced to undergo apoptosis by serum deprivation with survival rates of 48% (no insert), 51% (LacZ) and 45% (antisense NAIP) at 48 hours (Fig. 1a). In contrast, CHO cells infected with adenovirus expressing NAIP demonstrate 78-83% survival. NAIP also induced survival in stably transfected CHO pools, albeit slightly less than that seen in adenovirus infected cells: 44% of the vector transfectants and 65% of the NAIP transfectants survived at 48 hours (Fig. 1b). Next, overexpression of NAIP in CHO cells treated with 20 μM menadione (a potent inducer of free radicals) resulted in 20-30% enhancement of survival compared with controls after 24 hours (Figs. 1c, 1d). Overexpression of NAIP also protected menadione treated Rat-1 fibroblasts from undergoing cell death (Figs. 1e, 1f, 1g, 1h). Only 15% of cells infected with LacZ expressing adenovirus were viable at 12 hours in contrast to 80% of NAIP infected cells, an effect also detected with the pooled Rat-1 NAIP transfectants. Even greater survival was induced by NAIP overexpression at a lower menadione concentration (5μM), with 98% of pooled NAIP transfectants and 33% of control transfectants viable at 24 hours (Figs. 1g, 1h). Also assessed was the protective effect of NAIP on cells exposed to the cytokine TNF-α. HeLa cells treated with TNF-α and cyclohexamide were protected from apoptosis when infected with adenovirus expressing high levels

of NAIP (139%) at 48 hours, an effect not observed with antisense NAIP (52%) (Figs. 1i, 1j). A similar effect was observed in pooled HeLa transformants.

To confirm that cells surviving the apoptotic agents expressed NAIP, immunofluorescence with anti-NAIP antisera was performed on a number of the cell death assays. Immunofluorescence is a technique which localizes proteins within a cell by light microscopy by the use of antibodies specific for a desired protein and a fluorescence microscope. Dyes can be chemically coupled to antibodies directed against purified antibodies specific for a desired protein. This flourescent dye-antibody complex when added to permeabilized cells or tissue sections binds to the desired antigen-antibody which lights up when illuminated by the exciting wavelength. Fluorescent antibodies may also be microinjected into cultured cells for visualization. Using immunofluorescence, CY-3, a dye which emits red light, was coupled to a secondary antibody used to detect the bount anti-NAIP antibodies. A dramatic enrichment of NAIP expressing cells was observed, with no alteration noted in the cytoplasmic distribution of NAIP. These data offer strong support for the apoptotic suppression activity of NAIP.

Example 6 Cellular Distribution of NAIP using NAIP Antibodies

It was previously demonstrated (Roy, N. et. al. The gene for NAIP, a novel protein with homology to baculoviral inhibitor of apoptosis, is partially deleted in individuals with spinal muscle atrophy. *Cell* 80: 167-178 (1995).) by reverse transcriptase PCR analysis that the NAIP transcript is present in human spinal cord. To define more precisely the cellular distribution of NAIP, a polyclonal antiserum was raised against NAIP. The NAIP antibodies were then used in both immunocytochemistry and immunofluorescence techniques to visualize the protein directly in cells and tissues in order to establish the subcellular location and tissue specificity of the protein.

The ability of the polyclonal antibody to detect NAIP was confirmed by immunofluorescence of cells transfected with myc tagged NAIP employed both the anti-NAIP and anti-Myc antibodies, as well as western blot analysis on protein extracts of these cells (Fig. 1). In the western blotting technique, proteins are run on polyacrylamide gel and then transferred onto nitrocellulose membranes. These membranes are then incubated in the presence of the antibody

(primary), then following washing are incubated to a secondary antibody which is used for detection of the protein-primary antibody complex. Following repeated washing, the entire complex is visualized using colorimetric or chemiluminescent methods. A protein of the expected molecular weight was detected by both antibodies in western blots and their cellular co-localization demonstrated by immunofluorescence. Sections of human spinal cord stained with anti-NAIP showed strong immunoreactivity in the cytoplasm of the anterior horn cells and intermediolateral neurons (Figs. 3a and 3b). Consistent with the motor neuron staining, NAIP reactivity was observed in the ventral roots which contain motor axons but not the dorsal roots comprised of sensory axons (Figs. 3c and 3d). The observation of motor neuron staining correlates well with a role for the protein in the pathogenesis of SMA. However, the presence of NAIP in intermediolateral neurons which are not reported to be affected in SMA, implies heterogeneity in the apoptotic pathways between the two classes of neurons.

Other Embodiments

In other embodiments, the invention includes any protein which is substantially identical to a mammalian NAIP polypeptides provided in Figs. 6 and 7, Seq. ID NOS: 22 and 24); such homologs include other substantially pure naturally-occurring mammalian NAIP proteins as well as allelic variants; natural mutants; induced mutants; DNA sequences which encode proteins and also hybridize to the NAIP DNA sequences of Figs. 6 and 7, (Seq. ID NOS: 21 and 23) under high stringency conditions or, less preferably, under low stringency conditions (e.g., washing at 2X SSC at 400C with a probe length of at least 40 nucleotides); and proteins specifically bound by antisera directed to a NAIP polypeptide. The term also includes chimeric polypeptides that include a NAIP portion. The sequence of Seq. ID No. 1 and the IAP proteins are specifically excluded.

The invention further includes analogs of any naturally-occurring NAIP polypeptide. Analogs can differ from the naturally-occurring NAIP protein by amino acid sequence differences, by post-translational modifications, or by both. Analogs of the invention will generally exhibit at least 85%, more preferably 90%, and most preferably 95% or even 99% identity with all or part of a naturally occurring NAIP amino acid sequence. The length of sequence comparison is at least 15

amino acid residues, preferably at least 25 amino acid residues, and more preferably more than 35 amino acid residues. Modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, e.g., acetylation, carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-occurring NAIP polypeptide by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, 1989, or Ausubel et al., *supra*). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, e.g., D-amino acids or nonnaturally occurring or synthetic amino acids, e.g., B or y amino acids. In addition to full-length polypeptides, the invention also includes NAIP polypeptide fragments. As used herein, the term "fragment," means at least 20 contiguous amino acids, preferably at least 30 contiguous amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least 60 to 80 or more contiguous amino acids. Fragments of NAIP polypeptides can be generated by methods known to those skilled in the art or may result from normal protein processing (e.g., removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events).

Preferable fragments or analogs according to the invention are those which facilitate specific detection of a NAIP nucleic acid or amino acid sequence in a sample to be diagnosed. Particularly useful NAIP fragments for this purpose include, without limitation, the amino acid fragments shown in Table 2.

What is claimed is:

1. A method of inhibiting apoptosis in a cell, said method comprising administering to said cell an apoptosis inhibiting amount of NAIP polypeptide.

2. A method of inhibiting apoptosis in a mammal, said method comprising providing a transgene encoding a NAIP polypeptide or fragment thereof to a cell of said mammal, said transgene being positioned for expression in said cell.

3. A method of inhibiting apoptosis in a cell, said method comprising administering a compound which increases NAIP biological activity.

4. The method of claim 2, or 3 wherein said cell is in a mammal.

5. The method of claim 4, wherein said mammal is a human.

6. The method of claim 1 or 2, wherein said cell is in a mammal diagnosed as being HIV-positive, or as having AIDS, a neurodegenerative disease, a myelodysplastic syndrome, or an ischemic injury.

7. The method of claim 6, wherein said ischemic injury is caused by a myocardial infarction, a stroke, a reperfusion injury, or a toxin-induced liver disease, physical injury, renal failure, a secondary exsaunguination or blood flow interruption resulting from any other primary diseases.

8. The method of claim 1, 2, or 3, wherein said cell is a muscle cell.

9. The method of claim 1 or 2, wherein said muscle cell is a myocardial cell.

10. The method of claim 1 or 2, wherein said muscle cell is a renal cell.

11. The method of claim 1 or 2, wherein said muscle cell is a neuron.

12. The method of claim 2 wherein said transgene encodes NAIP.

13. The method of claim 6, wherein said mammal is HIV-positive or has AIDS.

14. The method of claim 13, wherein said cell is a T cell.

15. The method of claim 14, wherein said T cell is a $CD4^+$ T cell.

16. The method of claim 6, wherein said mammal has a neurodegenerative disease.

17. The method of claim 6, wherein said mammal has an ischemic injury.

18. A method for increasing apoptosis in a cell, said method comprising administering a compound which decreases NAIP anti-apoptotic activity.

19. The method of claim 18, wherein said compound is NAIP antisense RNA.

20. The method of claim 18, wherein said compound is an antibody which specifically binds NAIP.

21. A substantially pure nucleic acid encoding a NAIP polypeptide.

22. The nucleic acid of claim 21, wherein said nucleic acid is mammalian.

23. The nucleic acid of claim 22, wherein said mammal is a human.

24. The nucleic acid of claim 21, wherein said nucleic acid is genomic DNA or cDNA.

25. A substantially pure DNA having the sequence of Fig. 6, or degenerate variants thereof, and encoding the amino acid sequence of Fig. 6.

26. Substantially pure DNA having about 50% or greater nucleotide sequence identity to the DNA sequence of Fig. 6.

27. The DNA of claim 26, wherein said nucleotide sequence identity is 75% or greater.

28. A purified DNA sequence substantially identical to the DNA sequence shown in Fig. 6.

29 The DNA of claim 21, wherein said DNA is operably linked to regulatory sequences for expression of said polypeptide and wherein said regulatory sequences comprise a promoter.

30. The DNA of claim 29, wherein said promoter is a constitutive promoter, is inducible by one or more external agents, or is cell-type specific.

31. The nucleic acid of claim 21, wherein said nucleic acid comprises a deletion of the nucleic acids encoding the carboxy terminal amino acids of NAIP.

32. A vector comprising the nucleic acid of claim 21, said vector being capable of directing expression of the peptide encoded by said nucleic acid in a vector-containing cell.

33. A cell that contains the DNA of claim 21.

34. The cell of claim 33, said cell being present in a patient having a disease that is caused by excessive or insufficient cell death.

35. The cell of claim 33, said cell being selected from the group consisting of a fibroblast, a neuron, a glial cell, an insect cell, an embryonic stem cell, a myocardial cell, and a lymphocyte.

36. A transgenic cell that contains the DNA of claim 21, wherein said DNA is expressed in said transgenic cell.

37. A transgenic animal generated from the cell of claim 33, wherein said DNA is expressed in said transgenic animal.

38. A substantially pure mammalian NAIP polypeptide, or fragment thereof.

39. The fragment of claim 38, wherein said fragment comprises the three BIR domains of NAIP and lacks at least a portion of the carboxy terminus of NAIP.

40. The polypeptide of claim 38, said polypeptide being encoded by the nucleic acid of claim 17.

41. The polypeptide of claim 38, said polypeptide comprising an amino acid sequence substantially identical to an amino acid sequence shown in Figs. 6 or 7.

42. The polypeptide of claim 38, wherein said polypeptide is a mammalian polypeptide.

43. The polypeptide of claim 38, wherein said polypeptide is a human polypeptide.

44. A therapeutic composition comprising as an active ingredient a NAIP polypeptide according to claim 38, said active ingredient being formulated in a physiologically acceptable carrier.

45. The composition of claim 44, said active ingredient being a NAIP polypeptide encoded by the nucleic acid of claim 17.

46. A method of detecting a NAIP gene in an animal cell, said method comprising contacting the nucleic acid of claim 17, or a portion thereof that is greater than about 18 nucleotides in length, with a preparation of genomic DNA from said animal cell, said method providing detection of DNA sequences having about 50% or greater nucleotide sequence identity with the sequence of Fig. 6.

47. The method of claim 46, wherein said detecting is to diagnose a condition involving altered levels of apoptosis.

48. The method of claim 47, wherein said condition is Amyotrophic Lateral Sclerosis.

49. A method of obtaining a NAIP polypeptide, said method comprising:

(a) providing a cell with DNA encoding a NAIP polypeptide, said DNA being positioned for expression in said cell;

(b) culturing said cell under conditions for expressing said DNA; and

(c) isolating said NAIP polypeptide.

50. The method of claim 49, wherein said DNA further comprises a promotor inducible by one or more external agents.

51. A method of isolating a NAIP gene or portion thereof having sequence identity to human NAIP, said method comprising amplifying by PCR said NAIP gene or portion thereof using oligonucleotide primers wherein said primers

(a) are each greater than 13 nucleotides in length;

(b) each have regions of complementarity to opposite DNA strands in a region of the nucleotide sequence of either Fig. 6; and

(c) optionally contain sequences capable of producing restriction enzyme cut sites in the amplified product; and isolating said NAIP gene or portion thereof.

52. A method of isolating a NAIP gene or fragment thereof from a cell, said method comprising:

(a) providing a sample of cellular DNA;

(b) providing a pair of oligonucleotides having sequence homology to a conserved region of a NAIP gene;

(c) combining said pair of oligonucleotides with said cellular DNA sample under conditions suitable for polymerase chain reaction-mediated DNA amplification; and

(d) isolating said amplified NAIP gene or fragment thereof.

53. The method of claim 52, wherein said amplification is carried out using a reverse-transcription polymerase chain reaction.

54. The method of claim 53, wherein said reverse-transcription polymerase chain reaction is RACE.

55. A method of identifying a NAIP gene in a mammalian cell, said method comprising:

(a) providing a preparation of mammalian cellular DNA;

(b) providing a detectably-labelled DNA sequence having homology to a conserved region of a NAIP gene;

(c) contacting said preparation of cellular DNA with said detectably-labelled DNA sequence under hybridization conditions that provide detection of genes having 50% or greater nucleotide sequence identity; and

56. The method of claim 51, 52, or 55 wherein said DNA sequence comprises at least a portion of exon 14a or exon 17 of NAIP.

57. A NAIP gene isolated according to a method comprising:

(a) providing a sample of cellular DNA;

(b) providing DNA sequence, said sequence comprising a pair of oligonucleotides having sequence homology to a conserved region of a NAIP gene absent in Seq. ID No. 1;

(c) combining said pair of oligonucleotides with said cellular DNA sample under conditions suitable for polymerase chain reaction-mediated DNA amplification; and

(d) isolating said amplified NAIP gene or fragment thereof.

58. A NAIP gene isolated according to the method comprising:

(a) providing a preparation of cellular DNA;

(b) providing a detectably-labelled DNA sequence having homology to a conserved region of a NAIP gene absent in Seq. ID No. 1;

(c) contacting said preparation of cellular DNA with said detectably-labelled DNA sequence under hybridization conditions providing detection of genes having 50% or greater nucleotide sequence identity; and

(d) identifying a NAIP gene by its association with said detectable label.

59. A method of identifying a NAIP gene, said method comprising:

(a) providing a mammalian cell sample;

(b) introducing by transformation into said cell sample a candidate NAIP gene;

(c) expressing said candidate NAIP gene within said cell sample; and

(d) determining whether said sample exhibits an altered level of apoptosis whereby an alteration in the level of apoptosis identifies a NAIP gene.

60. The method of claim 59, wherein said cell sample is selected from the group consisting of a lymphocyte, a fibroblast, an insect cell, a glial cell, a myocardial cell, an embryonic stem cell, and a neuron.

61. A purified antibody that binds specifically to a NAIP polypeptide.

62. A method of identifying a compound that modulates apoptosis, said method comprising:

(a) providing a cell expressing a NAIP polypeptide; and

(b) contracting said cell with a candidate compound and monitoring the expression of a NAIP gene, an alteration in the level of expression of said gene indicating the presence of a compound which modulates apoptosis.

63. The method of claim 62, wherein said NAIP gene is human NAIP.

64. The method of claim 63, wherein said cell is a myocardial cell expression.

65. A method of diagnosing a mammal for the presence of disease involving altered apoptosis or an increased likelihood of developing a disease involving altered apoptosis, said method comprising isolating a sample of nucleic acid from said mammal and determining whether said nucleic acid comprises a NAIP mutation, said mutation being an indication that said mammal has an apoptosis disease or an increased likelihood of developing a disease involving apoptosis.

66. A method of diagnosing a mammal for the presence of a disease involving altered apoptosis or an increased likelihood of developing a disease involving altered apoptosis, said method comprising measuring NAIP gene expression in a sample from said mammal, an alteration in said expression relative to a sample from an unaffected mammal being an indication that said mammal has an apoptosis disease or increased likelihood of developing an apoptosis disease.

67. The method of claim 65, wherein said NAIP gene is human NAIP.

68. The method of claim 65, wherein said gene expression is measured by assaying the amount of NAIP polypeptide in said sample.

69. The method of claim 66, wherein said NAIP polypeptide is measured by immunological methods or by assaying the amount of NAIP RNA in said sample.

70. A kit for diagnosing a mammal for the presence of a disease involving altered apoptosis or an increased likelihood of developing a disease involving altered apoptosis, said kit comprising a substantially pure antibody that specifically binds a NAIP polypeptide.

71. The kit of claim 70, further comprising a means for detecting said binding of said antibody to said NAIP polypeptide.

72. A method of inducing apoptosis in a cell, said method comprising administering to said cell a negative regulator of the NAIP-dependent anti-apoptotic pathway.

73. The method of claim 72, wherein said negative regulator is a purified antibody or a fragment thereof that binds specifically to a NAIP polypeptide.

74. The method of claim 73, wherein said negative regulator is a NAIP antisense mRNA molecule.

75. A NAIP nucleic acid for use in modulating apoptosis.

76. A NAIP polypeptide for use in modulating apoptosis.

77. The use of a NAIP polypeptide for the manufacture of a medicament for the modulation of apoptosis.

78. The use of a NAIP nucleic acid for the manufacture of a medicament for the modulation of apoptosis.

79. A method of treating SMA in a patient, said method comprising administering a polypeptide having at least two BIR domains of an anti-apoptotic protein.

80. A method of treating SMA in a patient, said method comprising administering a nucleic acid encoding a polypeptide having at least two BIR domains of an anti-apoptotic protein.

81. The method of claim 79 or 80, wherein said polypeptide has at least three BIR domains.

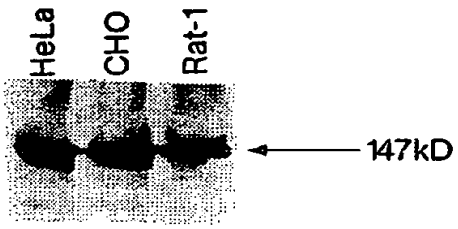


Fig. 1A

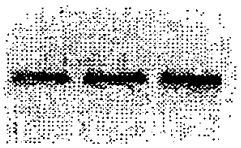


Fig. 1B

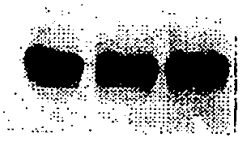


Fig. 1C

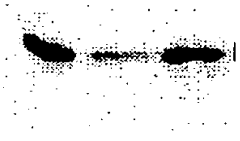


Fig. 1D

Fig. 1E

Fig. 1F

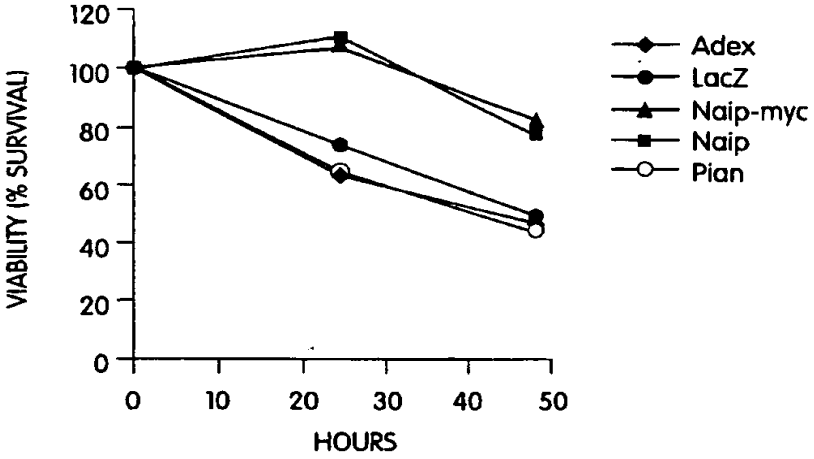
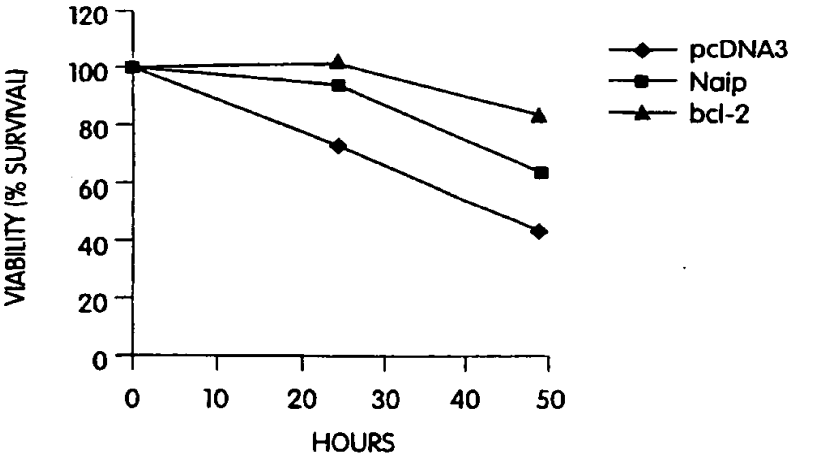

VIABILITY (% SURVIVAL)
120
100
80
60
40
20
0
0
10
20
30
40
50
HOURS
Adex
LacZ
Naip-myc
Naip
Pian

Fig. 2a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100
80
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0
10
20
30
40
50
HOURS
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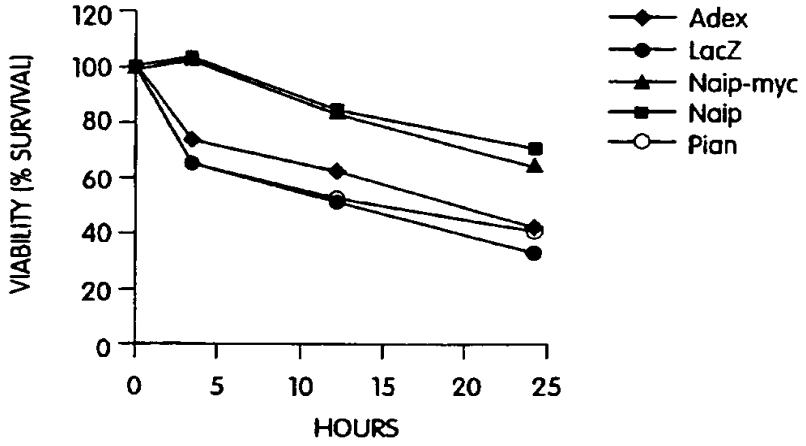
Naip
bcl-2

Fig. 2b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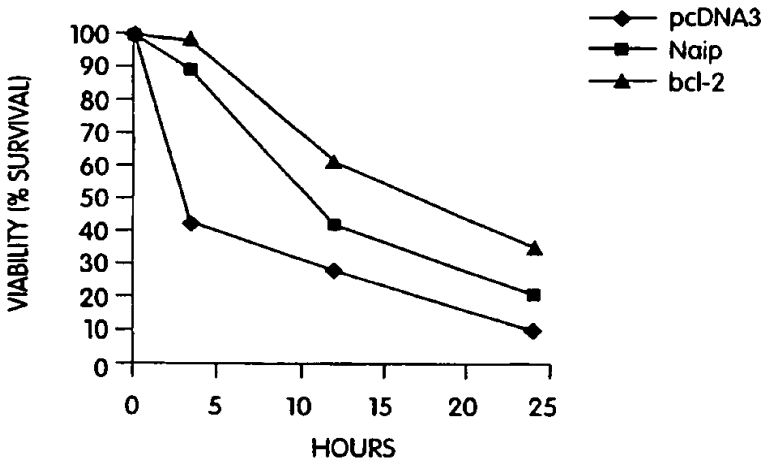
100
80
60
40
20
0
0
5
10
15
20
25
HOURS
Adex
LacZ
Naip-myc
Naip
Pian

Fig. 2c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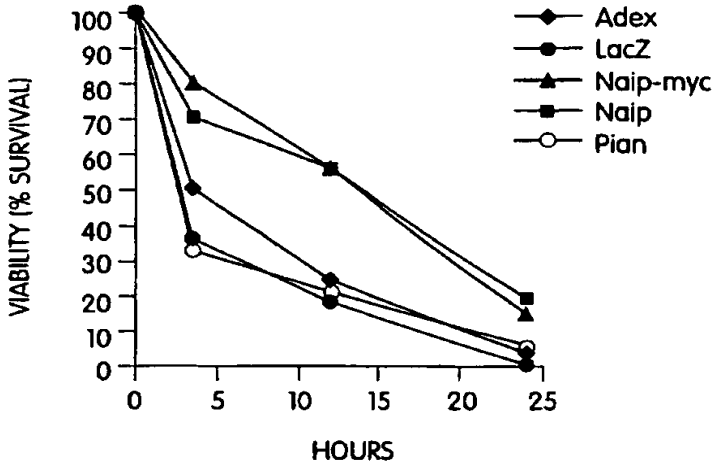
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
HOURS
pcDNA3
Naip
bcl-2

Fig. 2d


VIABILITY (% SURVIVAL)
100
90
80
70
60
50
40
30
20
10
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5
10
15
20
25
HOURS
Adex
LacZ
Naip-myc
Naip
Pian

Fig. 2e

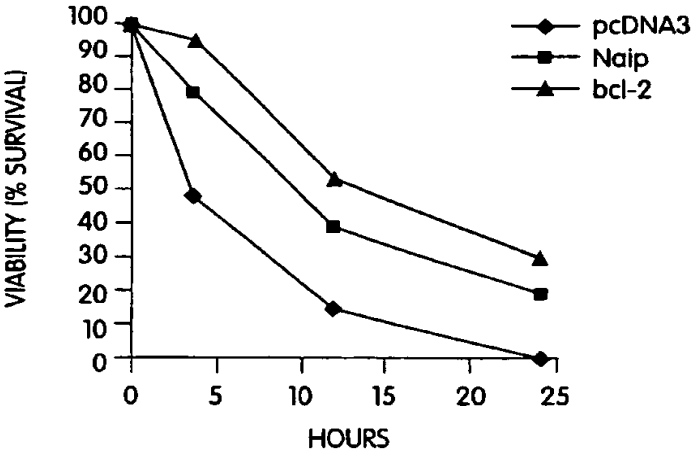
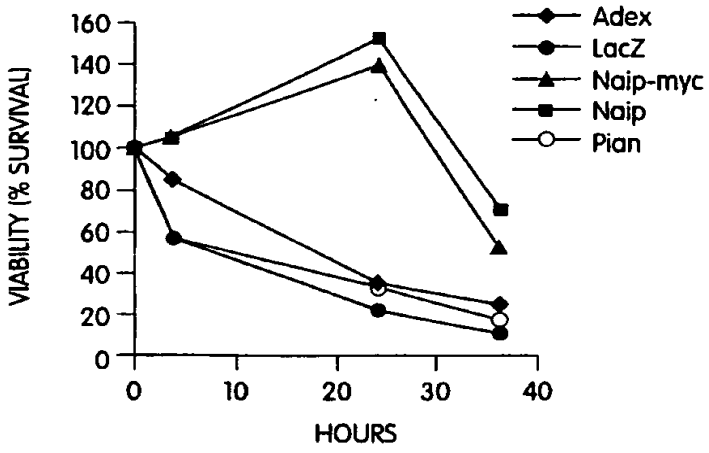

VIABILITY (% SURVIVAL)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
HOURS
pcDNA3
Naip
bcl-2

Fig. 2f


Adex
LacZ
Naip-myc
Naip
Pian
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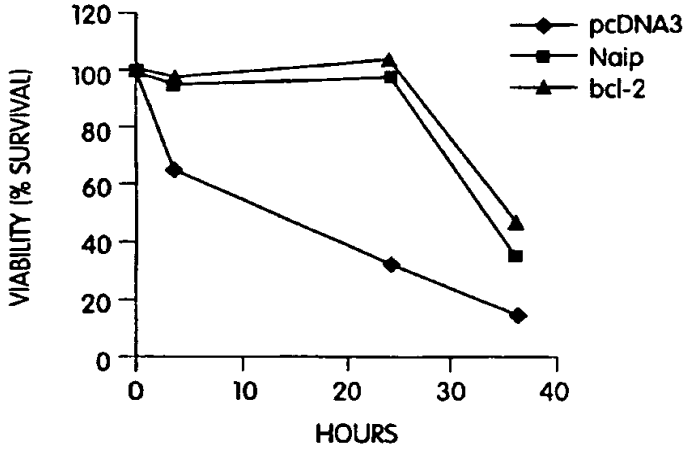
140
120
100
80
60
40
20
0
0
10
20
30
40
HOURS

Fig. 2g


pcDNA3
Naip
bcl-2
VIABILITY (% SURVIVAL)
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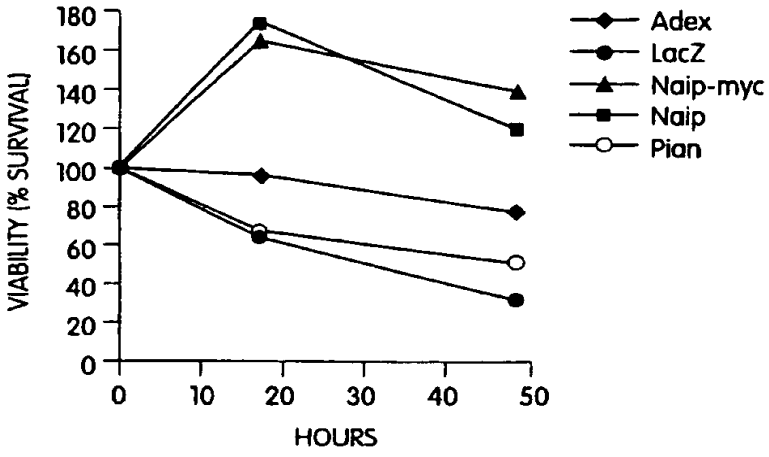
100
80
60
40
20
0
0
10
20
30
40
HOURS

Fig. 2h


VIABILITY (% SURVIVAL)
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160
140
120
100
80
60
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20
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10
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40
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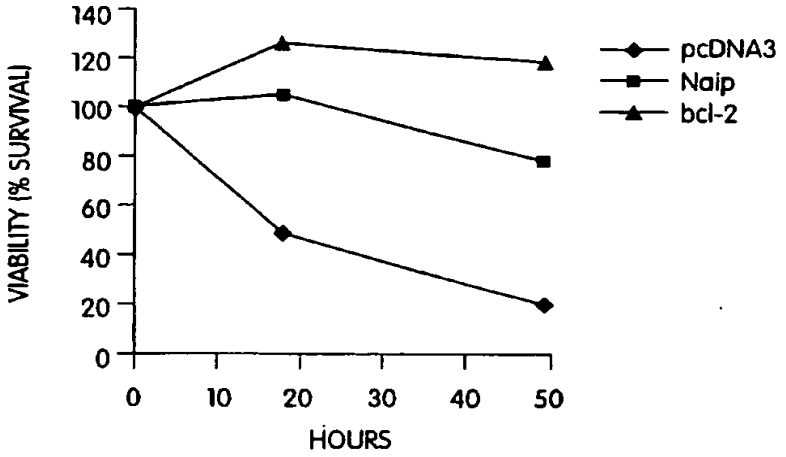
HOURS
Adex
LacZ
Naip-myc
Naip
Pian

Fig. 2i


VIABILITY (% SURVIVAL)
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120
100
80
60
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0
10
20
30
40
50
HOURS
pcDNA3
Naip
bcl-2

Fig. 2j

Fig. 3A

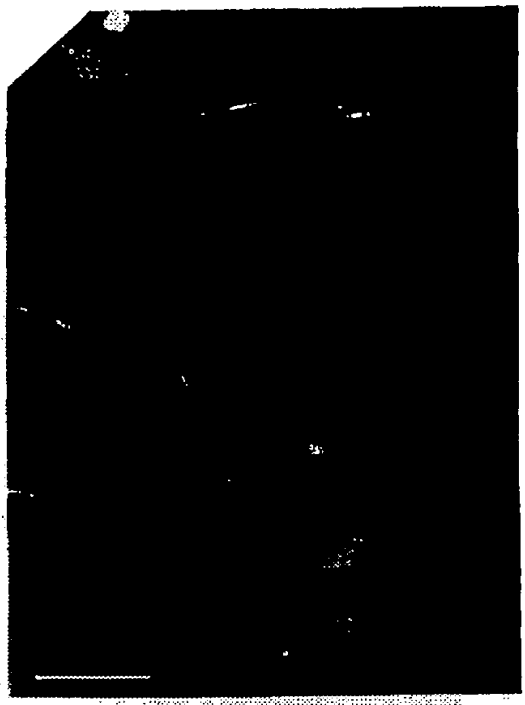

Fig. 3B

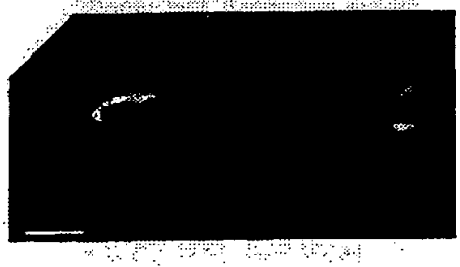

Fig. 3C

Fig. 3D

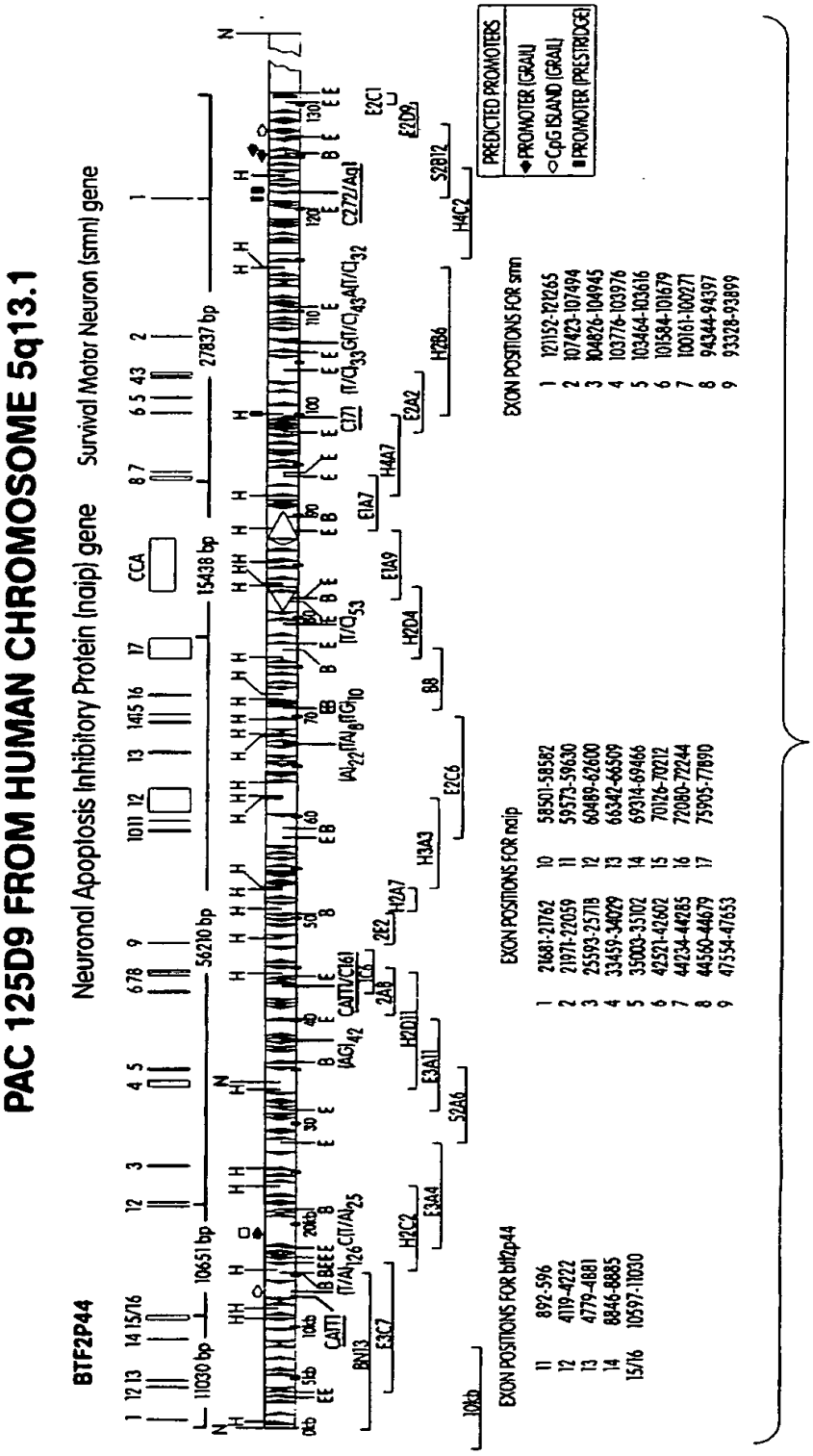
PAC 125D9 FROM HUMAN CHROMOSOME 5q13.1
Neuronal Apoptosis Inhibitory Protein (naip) gene
Survival Motor Neuron (smn) gene
BTF2P44
PREDICTED PROMOTERS
PROMOTER (GRAIL)
CpG ISLAND (GRAIL)
PROMOTER (PRESTRIDGE)
EXON POSITIONS FOR smn
1 121152-121265
2 107423-107494
3 104826-104945
4 103776-103976
5 103464-103616
6 101584-101679
7 100161-100271
8 94344-94397
9 93328-93899
EXON POSITIONS FOR naip
1 21681-21762
2 21971-22059
3 25593-25718
4 33459-34029
5 35003-35102
6 42521-42602
7 44234-44285
8 44560-44679
9 47554-47653
10 58501-58582
11 59573-59630
12 60489-62600
13 66342-66509
14 69314-69466
15 70126-70212
16 72080-72244
17 75905-77890
EXON POSITIONS FOR btf2p44
11 892-596
12 4119-4222
13 4779-4881
14 8846-8885
15/16 10597-11030
11030 bp
10651 bp
5621 0 bp
15438 bp
27837 bp

Fig. 4

```
>HSU19251, 5502 bases, 79F5B1F2 checksum.        5502 nt vs.
>naip.seq, 6133 bases, FD809D8 checksum.         6133 nt
77.8% identity; Optimized score: 13374

                10        20        30        40        50        60
naip-o TTCCGGCTGGACGTTGCCCTGTGTACCTCTTCGACTGCCTGTTCATCTACGACGAACCCC
       :
naip.s T-----------------------------------------------------------

                70        80        90       100       110       120
naip-o GGGTATTGACCCCAGACAACAATGCCACTTCATATTGCATGAAGACAAAAGGTCCTGTGC
                                                 ::::::::::::::::::::::::
naip.s -----------------------------------GCATGAAGACAAAAGGTCCTGTGC
                                                      10        20

              130       140       150       160       170       180
naip-o TCACCTGGGACCCTTCTGGACGTTGCCCTGTGTTCCTCTTCGCCTGCCTGTTCATCTACG
       :::::::::::::::::::::::::::::::::: :::::::: ::::::::::::::::::
naip.s TCACCTGGGACCCTTCTGGACGTTGCCCTGTGTACCTCTTCGACTGCCTGTTCATCTACG
          30        40        50        60        70        80

              190       200       210       220       230       240
naip-o ACGAACCCCGGGTATTGACCCCAGACAACAATGCCACTTCATATTGGGGACTTCGTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ACGAACCCCGGGTATTGACCCCAGACAACAATGCCACTTCATATTGGGGACTTCGTCTGG
          90       100       110       120       130       140

              250       260       270       280       290       300
naip-o GATTCCAAGGTGCATTCATTGCAAAGTTCCTTAAATATTTTCTCACTGCTTCCTACTAAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s GATTCCAAGGTGCATTCATTGCAAAGTTCCTTAAATATTTTCTCACTGCTTCCTACTAAA
         150       160       170       180       190       200

              310       320       330       340       350       360
naip-o GGACGGACAGAGCATTTGTTCTTCAGCCACATACTTTCCTTCCACTGGCCAGCATTCTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s GGACGGACAGAGCATTTGTTCTTCAGCCACATACTTTCCTTCCACTGGCCAGCATTCTCC
         210       220       230       240       250       260

              370       380       390       400       410       420
naip-o TCTATTAGACTAGAACTGTGGATAAACCTCAGAAAATGGCCACCCAGCAGAAAGCCTCTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s TCTATTAGACTAGAACTGTGGATAAACCTCAGAAAATGGCCACCCAGCAGAAAGCCTCTG
         270       280       290       300       310       320

              430       440       450       460       470       480
naip-o ACGAGAGGATCTCCCAGTTTGATCACAATTTGCTGCCAGAGCTGTCTGCTCTTCTGGGCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ACGAGAGGATCTCCCAGTTTGATCACAATTTGCTGCCAGAGCTGTCTGCTCTTCTGGGCC
         330       340       350       360       370       380
```

Fig. 5A

```
                490        500        510        520        530        540
naip-o  TAGATGCAGTTCAGTTGGCAAAGGAACTAGAAGAAGAGGAGCAGAAGGAGCGAGCAAAAA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s  TAGATGCAGTTCAGTTGGCAAAGGAACTAGAAGAAGAGGAGCAGAAGGAGCGAGCAAAAA
          390        400        410        420        430        440

                550        560        570        580        590        600
naip-o  TGCAGAAAGGCTACAACTCTCAAATGCGCAGTGAAGCAAAAAGGTTAAAGACTTTTGTGA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s  TGCAGAAAGGCTACAACTCTCAAATGCGCAGTGAAGCAAAAAGGTTAAAGACTTTTGTGA
          450        460        470        480        490        500

                610        620        630        640        650        660
naip-o  CTTATGAGCCGTACAGCTCATGGATACCACAGGAGATGGCGGCCGCTGGGTTTTACTTCA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s  CTTATGAGCCGTACAGCTCATGGATACCACAGGAGATGGCGGCCGCTGGGTTTTACTTCA
          510        520        530        540        550        560

                670        680        690        700        710        720
naip-o  CTGGGGTAAAATCTGGGATTCAGTGCTTCTGCTGTAGCCTAATCCTCTTTGGTGCCGGCC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s  CTGGGGTAAAATCTGGGATTCAGTGCTTCTGCTGTAGCCTAATCCTCTTTGGTGCCGGCC
          570        580        590        600        610        620

                730        740        750        760        770        780
naip-o  TCACGAGACTCCCCATAGAAGACCACAAGAGGTTTCATCCAGATTGTGGGTTCCTTTTGA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s  TCACGAGACTCCCCATAGAAGACCACAAGAGGTTTCATCCAGATTGTGGGTTCCTTTTGA
          630        640        650        660        670        680

                790        800        810        820        830        840
naip-o  ACAAGGATGTTGGTAACATTGCCAAGTACGACATAAGGGTGAAGAATCTGAAGAGCAGGC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s  ACAAGGATGTTGGTAACATTGCCAAGTACGACATAAGGGTGAAGAATCTGAAGAGCAGGC
          690        700        710        720        730        740

                850        860        870        880        890        900
naip-o  TGAGAGGAGGTAAAATGAGGTACCAAGAAGAGGAGGCTAGACTTGCATCCTTCAGGAACT
        :::::::::::::::::::::::::::::::::::::::::: ::::::::::::::::::
naip.s  TGAGAGGAGGTAAAATGAGGTACCAAGAAGAGGAGGCTAGACTTGCGTCCTTCAGGAACT
          750        760        770        780        790        800

                910        920        930        940        950        960
naip-o  GGCCATTTTATGTCCAAGGGATATCCCCTTGTGTGCTCTCAGAGGCTGGCTTTGTCTTTA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s  GGCCATTTTATGTCCAAGGGATATCCCCTTGTGTGCTCTCAGAGGCTGGCTTTGTCTTTA
          810        820        830        840        850        860

                970        980        990       1000       1010       1020
naip-o  CAGGTAAACAGGACACGGTACAGTGTTTTTCCTGTGGTGGATGTTTAGGAAATTGGGAAG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s  CAGGTAAACAGGACACGGTACAGTGTTTTTCCTGTGGTGGATGTTTAGGAAATTGGGAAG
          870        880        890        900        910        920
```

Fig. 5B

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          1030       1040       1050       1060       1070       1080
naip-o AAGGAGATGATCCTTGGAAGGAACATGCCAAATGGTTCCCCAAATGTGAATTTCTTCGGA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AAGGAGATGATCCTTGGAAGGAACATGCCAAATGGTTCCCCAAATGTGAATTTCTTCGGA
         930        940        950        960        970        980

          1090       1100       1110       1120       1130       1140
naip-o GTAAGAAATCCTCAGAGGAAATTACCCAGTATATTCAAAGCTACAAGGGATTTGTTGACA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s GTAAGAAATCCTCAGAGGAAATTACCCAGTATATTCAAAGCTACAAGGGATTTGTTGACA
         990       1000       1010       1020       1030       1040

          1150       1160       1170       1180       1190       1200
naip-o TAACGGGAGAACATTTTGTGAATTCCTGGGTCCAGAGAGAATTACCTATGGCATCAGCTT
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s TAACGGGAGAACATTTTGTGAATTCCTGGGTCCAGAGAGAATTACCTATGGCATCAGCTT
        1050       1060       1070       1080       1090       1100

          1210       1220       1230       1240       1250       1260
naip-o ATTGCAATGACAGCATCTTTGCTTACGAAGAACTACGGCTGGACTCTTTTAAGGACTGGC
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ATTGCAATGACAGCATCTTTGCTTACGAAGAACTACGGCTGGACTCTTTTAAGGACTGGC
        1110       1120       1130       1140       1150       1160

          1270       1280       1290       1300       1310       1320
naip-o CCCGGGAATCAGCTGTGGGAGTTGCAGCACTGGCCAAAGCAGGTCTTTTCTACACAGGTA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s CCCGGGAATCAGCTGTGGGAGTTGCAGCACTGGCCAAAGCAGGTCTTTTCTACACAGGTA
        1170       1180       1190       1200       1210       1220

          1330       1340       1350       1360       1370       1380
naip-o TAAAGGACATCGTCCAGTGCTTTTCCTGTGGAGGGTGTTTAGAGAAATGGCAGGAAGGTG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s TAAAGGACATCGTCCAGTGCTTTTCCTGTGGAGGGTGTTTAGAGAAATGGCAGGAAGGTG
        1230       1240       1250       1260       1270       1280

          1390       1400       1410       1420       1430       1440
naip-o ATGACCCATTAGACGATCACACCAGATGTTTTCCCAATTGTCCATTTCTCCAAAATATGA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ATGACCCATTAGACGATCACACCAGATGTTTTCCCAATTGTCCATTTCTCCAAAATATGA
        1290       1300       1310       1320       1330       1340

          1450       1460       1470       1480       1490       1500
naip-o AGTCCTCTGCGGAAGTGACTCCAGACCTTCAGAGCCGTGGTGAACTTTGTGAATTACTGG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AGTCCTCTGCGGAAGTGACTCCAGACCTTCAGAGCCGTGGTGAACTTTGTGAATTACTGG
        1350       1360       1370       1380       1390       1400

          1510       1520       1530       1540       1550       1560
naip-o AAACCACAAGTGAAAGCAATCTTGAAGATTCAATAGCAGTTGGTCCTATAGTGCCAGAAA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AAACCACAAGTGAAAGCAATCTTGAAGATTCAATAGCAGTTGGTCCTATAGTGCCAGAAA
        1410       1420       1430       1440       1450       1460
```

Fig. 5C

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          1570       1580       1590       1600       1610       1620
naip-o TGGCACAGGGTGAAGCCCAGTGGTTTCAAGAGGCAAAGAATCTGAATGAGCAGCTGAGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s TGGCACAGGGTGAAGCCCAGTGGTTTCAAGAGGCAAAGAATCTGAATGAGCAGCTGAGAG
        1470       1480       1490       1500       1510       1520

          1630       1640       1650       1660       1670       1680
naip-o CAGCTTATACCAGCGCCAGTTTCCGCCACATGTCTTTGCTTGATATCTCTTCCGATCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s CAGCTTATACCAGCGCCAGTTTCCGCCACATGTCTTTGCTTGATATCTCTTCCGATCTGG
        1530       1540       1550       1560       1570       1580

          1690       1700       1710       1720       1730       1740
naip-o CCACGGACCACTTGCTGGGCTGTGATCTGTCTATTGCTTCAAAACACATCAGCAAACCTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s CCACGGACCACTTGCTGGGCTGTGATCTGTCTATTGCTTCAAAACACATCAGCAAACCTG
        1590       1600       1610       1620       1630       1640

          1750       1760       1770       1780       1790       1800
naip-o TGCAAGAACCTCTGGTGCTGCCTGAGGTCTTTGGCAACTTGAACTCTGTCATGTGTGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s TGCAAGAACCTCTGGTGCTGCCTGAGGTCTTTGGCAACTTGAACTCTGTCATGTGTGTGG
        1650       1660       1670       1680       1690       1700

          1810       1820       1830       1840       1850       1860
naip-o AGGGTGAAGCTGGAAGTGGAAAGACGGTCCTCCTGAAGAAAATAGCTTTTCTGTGGGCAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AGGGTGAAGCTGGAAGTGGAAAGACGGTCCTCCTGAAGAAAATAGCTTTTCTGTGGGCAT
        1710       1720       1730       1740       1750       1760

          1870       1880       1890       1900       1910       1920
naip-o CTGGATGCTGTCCCCTGTTAAACAGGTTCCAGCTGGTTTTCTACCTCTCCCTTAGTTCCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s CTGGATGCTGTCCCCTGTTAAACAGGTTCCAGCTGGTTTTCTACCTCTCCCTTAGTTCCA
        1770       1780       1790       1800       1810       1820

          1930       1940       1950       1960       1970       1980
naip-o CCAGACCAGACGAGGGGCTGGCCAGTATCATCTGTGACCAGCTCCTAGAGAAAGAAGGAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s CCAGACCAGACGAGGGGCTGGCCAGTATCATCTGTGACCAGCTCCTAGAGAAAGAAGGAT
        1830       1840       1850       1860       1870       1880

          1990       2000       2010       2020       2030       2040
naip-o CTGTTACTGAAATGTGCATGAGGAACATTATCCAGCAGTTAAAGAATCAGGTCTTATTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s CTGTTACTGAAATGTGCATGAGGAACATTATCCAGCAGTTAAAGAATCAGGTCTTATTCC
        1890       1900       1910       1920       1930       1940

          2050       2060       2070       2080       2090       2100
naip-o TTTTAGATGACTACAAAGAAATATGTTCAATCCCTCAAGTCATAGGAAAACTGATTCAAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s TTTTAGATGACTACAAAGAAATATGTTCAATCCCTCAAGTCATAGGAAAACTGATTCAAA
        1950       1960       1970       1980       1990       2000
```

Fig. 5D

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          2110       2120       2130       2140       2150       2160
naip-o AAAACCACTTATCCCGGACCTGCCTATTGATTGCTGTCCGTACAAACAGGGCCAGGGACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AAAACCACTTATCCCGGACCTGCCTATTGATTGCTGTCCGTACAAACAGGGCCAGGGACA
        2010       2020       2030       2040       2050       2060

          2170       2180       2190       2200       2210       2220
naip-o TCCGCCGATACCTAGAGACCATTCTAGAGATCCAAGCATTTCCCTTTTATAATACTGTCT
       :::::::::::::::::::::::::::::::: ::::::::::::::::::::::::::
naip.s TCCGCCGATACCTAGAGACCATTCTAGAGATCAAAGCATTTCCCTTTTATAATACTGTCT
        2070       2080       2090       2100       2110       2120

          2230       2240       2250       2260       2270       2280
naip-o GTATATTACGGAAGCTCTTTTCACATAATATGACTCGTCTGCGAAAGTTTATGGTTTACT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s GTATATTACGGAAGCTCTTTTCACATAATATGACTCGTCTGCGAAAGTTTATGGTTTACT
        2130       2140       2150       2160       2170       2180

          2290       2300       2310       2320       2330       2340
naip-o TTGGAAAGAACCAAAGTTTGCAGAAGATACAGAAAACTCCTCTCTTTGTGGCGGCGATCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s TTGGAAAGAACCAAAGTTTGCAGAAGATACAGAAAACTCCTCTCTTTGTGGCGGCGATCT
        2190       2200       2210       2220       2230       2240

          2350       2360       2370       2380       2390       2400
naip-o GTGCTCATTGGTTTCAGTATCCTTTTGACCCATCCTTTGATGATGTGGCTGTTTTCAAGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s GTGCTCATTGGTTTCAGTATCCTTTTGACCCATCCTTTGATGATGTGGCTGTTTTCAAGT
        2250       2260       2270       2280       2290       2300

          2410       2420       2430       2440       2450       2460
naip-o CCTATATGGAACGCCTTTCCTTAAGGAACAAAGCGACAGCTGAAATTCTCAAAGCAACTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s CCTATATGGAACGCCTTTCCTTAAGGAACAAAGCGACAGCTGAAATTCTCAAAGCAACTG
        2310       2320       2330       2340       2350       2360

          2470       2480       2490       2500       2510       2520
naip-o TGTCCTCCTGTGGTGAGCTGGCCTTGAAAGGGTTTTTTTCATGTTGCTTTGAGTTTAATG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s TGTCCTCCTGTGGTGAGCTGGCCTTGAAAGGGTTTTTTTCATGTTGCTTTGAGTTTAATG
        2370       2380       2390       2400       2410       2420

          2530       2540       2550       2560       2570       2580
naip-o ATGATGATCTCGCAGAAGCAGGGGTTGATGAAGATGAAGATCTAACCATGTGCTTGATGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ATGATGATCTCGCAGAAGCAGGGGTTGATGAAGATGAAGATCTAACCATGTGCTTGATGA
        2430       2440       2450       2460       2470       2480

          2590       2600       2610       2620       2630       2640
naip-o GCAAATTTACAGCCCAGAGACTAAGACCATTCTACCGGTTTTTAAGTCCTGCCTTCCAAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s GCAAATTTACAGCCCAGAGACTAAGACCATTCTACCGGTTTTTAAGTCCTGCCTTCCAAG
        2490       2500       2510       2520       2530       2540
```

Fig. 5E

```
          2650       2660       2670       2680       2690       2700
naip-o AATTTCTTGCGGGGATGAGGCTGATTGAACTCCTGGATTCAGATAGGCAGGAACATCAAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AATTTCTTGCGGGGATGAGGCTGATTGAACTCCTGGATTCAGATAGGCAGGAACATCAAG
        2550       2560       2570       2580       2590       2600

          2710       2720       2730       2740       2750       2760
naip-o ATTTGGGACTGTATCATTTGAAACAAATCAACTCACCCATGATGACTGTAAGCGCCTACA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ATTTGGGACTGTATCATTTGAAACAAATCAACTCACCCATGATGACTGTAAGCGCCTACA
        2610       2620       2630       2640       2650       2660

          2770       2780       2790       2800       2810       2820
naip-o ACAATTTTTTGAACTATGTCTCCAGCCTCCCTTCAACAAAAGCAGGGCCCAAAATTGTGT
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ACAATTTTTTGAACTATGTCTCCAGCCTCCCTTCAACAAAAGCAGGGCCCAAAATTGTGT
        2670       2680       2690       2700       2710       2720

          2830       2840       2850       2860       2870       2880
naip-o CTCATTTGCTCCATTTAGTGGATAACAAAGAGTCATTGGAGAATATATCTGAAAATGATG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s CTCATTTGCTCCATTTAGTGGATAACAAAGAGTCATTGGAGAATATATCTGAAAATGATG
        2730       2740       2750       2760       2770       2780

          2890       2900       2910       2920       2930       2940
naip-o ACTACTTAAAGCACCAGCCAGAAATTTCACTGCAGATGCAGTTACTTAGGGGATTGTGGC
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ACTACTTAAAGCACCAGCCAGAAATTTCACTGCAGATGCAGTTACTTAGGGGATTGTGGC
        2790       2800       2810       2820       2830       2840

          2950       2960       2970       2980       2990       3000
naip-o AAATTTGTCCACAAGCTTACTTTTCAATGGTTTCAGAACATTTACTGGTTCTTGCCCTGA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AAATTTGTCCACAAGCTTACTTTTCAATGGTTTCAGAACATTTACTGGTTCTTGCCCTGA
        2850       2860       2870       2880       2890       2900

          3010       3020       3030       3040       3050       3060
naip-o AAACTGCTTATCAAAGCAACACTGTTGCTGCGTGTTCTCCATTTGTTTTGCAATTCCTTC
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AAACTGCTTATCAAAGCAACACTGTTGCTGCGTGTTCTCCATTTGTTTTGCAATTCCTTC
        2910       2920       2930       2940       2950       2960

          3070       3080       3090       3100       3110       3120
naip-o AAGGGAGAACACTGACTTTGGGTGCGCTTAACTTACAGTACTTTTTCGACCACCCAGAAA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AAGGGAGAACACTGACTTTGGGTGCGCTTAACTTACAGTACTTTTTCGACCACCCAGAAA
        2970       2980       2990       3000       3010       3020

          3130       3140       3150       3160       3170       3180
naip-o GCTTGTCATTGTTGAGGAGCATCCACTTCTCAATACGAGGAAATAAGACATCACCCAGAG
       :::::::::::::::::::::::::::::::: ::::::::::::::::::::::::::::
naip.s GCTTGTCATTGTTGAGGAGCATCCACTTCCCAATACGAGGAAATAAGACATCACCCAGAG
        3030       3040       3050       3060       3070       3080
```

Fig. 5F

```
            3190       3200       3210       3220       3230       3240
naip-o CACATTTTTCAGTTCTGGAAACATGTTTTGACAAATCACAGGTGCCAACTATAGATCAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s CACATTTTTCAGTTCTGGAAACATGTTTTGACAAATCACAGGTGCCAACTATAGATCAGG
        3090       3100       3110       3120       3130       3140

            3250       3260       3270       3280       3290       3300
naip-o ACTATGCTTCTGCCTTTGAACCTATGAATGAATGGGAGCGAAATTTAGCTGAAAAAGAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ACTATGCTTCTGCCTTTGAACCTATGAATGAATGGGAGCGAAATTTAGCTGAAAAAGAGG
        3150       3160       3170       3180       3190       3200

            3310       3320       3330       3340       3350       3360
naip-o ATAATGTAAAGAGCTATATGGATATGCAGCGCAGGGCATCACCAGACCTTAGTACTGGCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ATAATGTAAAGAGCTATATGGATATGCAGCGCAGGGCATCACCAGACCTTAGTACTGGCT
        3210       3220       3230       3240       3250       3260

            3370       3380       3390       3400       3410       3420
naip-o ATTGGAAACTTTCTCCAAAGCAGTACAAGATTCCCTGTCTAGAAGTCGATGTGAATGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ATTGGAAACTTTCTCCAAAGCAGTACAAGATTCCCTGTCTAGAAGTCGATGTGAATGATA
        3270       3280       3290       3300       3310       3320

            3430       3440       3450       3460       3470       3480
naip-o TTGATGTTGTAGGCCAGGATATGCTTGAGATTCTAATGACAGTTTTCTCAGCTTCACAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s TTGATGTTGTAGGCCAGGATATGCTTGAGATTCTAATGACAGTTTTCTCAGCTTCACAGC
        3330       3340       3350       3360       3370       3380

            3490       3500       3510       3520       3530       3540
naip-o GCATCGAACTCCATTTAAACCACAGCAGAGGCTTTATAGAAAGCATCCGCCCAGCTCTTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s GCATCGAACTCCATTTAAACCACAGCAGAGGCTTTATAGAAAGCATCCGCCCAGCTCTTG
        3390       3400       3410       3420       3430       3440

            3550       3560       3570       3580       3590       3600
naip-o AGCTGTCTAAGGCCTCTGTCACCAAGTGCTCCATAAGCAAGTTGGAACTCAGCGCAGCCG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AGCTGTCTAAGGCCTCTGTCACCAAGTGCTCCATAAGCAAGTTGGAACTCAGCGCAGCCG
        3450       3460       3470       3480       3490       3500

            3610       3620       3630       3640       3650       3660
naip-o AACAGGAACTGCTTCTCACCCTGCCTTCCCTGGAATCTCTTGAAGTCTCAGGGACAATCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AACAGGAACTGCTTCTCACCCTGCCTTCCCTGGAATCTCTTGAAGTCTCAGGGACAATCC
        3510       3520       3530       3540       3550       3560

            3670       3680       3690       3700       3710       3720
naip-o AGTCACAAGACCAAATCTTTCCTAATCTGGATAAGTTCCTGTGCCTGAAAGAACTGTCTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AGTCACAAGACCAAATCTTTCCTAATCTGGATAAGTTCCTGTGCCTGAAAGAACTGTCTG
        3570       3580       3590       3600       3610       3620
```

Fig. 5G

```
            3730       3740       3750       3760       3770       3780
naip-o TGGATCTGGAGGGCAATATAAATGTTTTTTCAGTCATTCCTGAAGAATTTCCAAACTTCC
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s TGGATCTGGAGGGCAATATAAATGTTTTTTCAGTCATTCCTGAAGAATTTCCAAACTTCC
        3630       3640       3650       3660       3670       3680

            3790       3800       3810       3820       3830       3840
naip-o ACCATATGGAGAAATTATTGATCCAAATTTCAGCTGAGTATGATCCTTCCAAACTAGTAA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ACCATATGGAGAAATTATTGATCCAAATTTCAGCTGAGTATGATCCTTCCAAACTAGTAA
        3690       3700       3710       3720       3730       3740

naip-o ------------------------------------------------------------

naip.s AATTAATTCAAAATTCTCCAAACCTTCATGTTTTCCATCTGAAGTGTAACTTCTTTTCGG
        3750       3760       3770       3780       3790       3800

naip-o ------------------------------------------------------------

naip.s ATTTTGGGTCTCTCATGACTATGCTTGTTTCCTGTAAGAAACTCACAGAAATTAAGTTTT
        3810       3820       3830       3840       3850       3860

                                        3840       3850       3860
naip-o ------------------------------TGCCAGTTTGCCAAATTTTATTTCTCTGA
                                      ::::::::::::::::::::::::::::
naip.s CGGATTCATTTTTTCAAGCCGTCCCATTTGTTGCCAGTTTGCCAAATTTTATTTCTCTGA
        3870       3880       3890       3900       3910       3920

      3870       3880       3890       3900       3910       3920
naip-o AGATATTAAATCTTGAAGGCCAGCAATTTCCTGATGAGGAAACATCAGAAAAATTTGCCT
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s AGATATTAAATCTTGAAGGCCAGCAATTTCCTGATGAGGAAACATCAGAAAAATTTGCCT
        3930       3940       3950       3960       3970       3980

      3930       3940       3950       3960       3970       3980
naip-o ACATTTTAGGTTCTCTTAGTAACCTGGAAGAATTGATCCTTCCTACTGGGGATGGAATTT
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ACATTTTAGGTTCTCTTAGTAACCTGGAAGAATTGATCCTTCCTACTGGGGATGGAATTT
        3990       4000       4010       4020       4030       4040

      3990       4000       4010       4020       4030       4040
naip-O ATCGAGTGGCCAAACTGATCATCCAGCAGTGTCAGCAGCTTCATTGTCTCCGAGTCCTCT
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ATCGAGTGGCCAAACTGATCATCCAGCAGTGTCAGCAGCTTCATTGTCTCCGAGTCCTCT
        4050       4060       4070       4080       4090       4100

      4050       4060       4070       4080       4090              4100
naip-O CATTTTTCAAGACTTTGAATGATGACAGCGTGGTGGAAATTGGTTAAA----AAT--GTG
       :::::::::::::::::::::::::::::::::::::::::::::::::  ::     :::  :::
naip.s CATTTTTCAAGACTTTGAATGATGACAGCGTGGTGGAAATTGCCAAAGTAGCAATCAGTG
        4110       4120       4130       4140       4150       4160
```

Fig. 5H

```
                4110        4120        4130        4140        4150
naip-o ----TCTGCAGGCACAC-AGGACGT---GCCTTCACCCC--CATCTGACTAT-GTGGAAA
          : : ::: :: :: :: :  :: :::: :  ::  :: ::  : ::::
naip.s GAGGTTTCCAGAAACTTGAGAACCTAAAGCTTTCAATCAATCACAAGATTACAGAGGAAG
        4170        4180        4190        4200        4210        4220

              4160        4170        4180        4190              4200
naip-o GAGTT-GACAGTCCCATGGCATACTCTTCCA-ATGGCAAAGT-----GAAT--GACAAGC
       ::    :: : : :  :      :::   : : ::: :::: :      :: :  ::::
naip.s GATACAGAAATTTCTTTCAAGCACTGGACAACATGCCAAACTTGCAGGAGTTGGACATCT
        4230        4240        4250        4260        4270        4280

             4210        4220          4230          4240
naip-o ---GGTTTTATCCAGAGTCTTCCTA---TAAATCCACGCCGGT----TCCTGAAGT----
         ::   ::    :::::: :  : :   : :  ::::  : ::     :: :  :::
naip.s CCAGGCATTTCACAGAGTGTATCAAAGCTCAGGCCACAACAGTCAAGTCTTTGAGTCAAT
        4290        4300        4310        4320        4330        4340

        4250        4260              4270        4280        4290
naip-o --GGTTCAGGAGCTTCCA-------TTA-ACTTCGCCTGTGGA--TGACTTCAGGCAGCC
         :  : : :: :: :::        ::: :::   : :::  :  :: ::   ::  ::
naip.s GTGTGTTACGA-CTACCAAGGCTCATTAGACTGAACATGTTAAGTTGGCTCTTGGATGCA
        4350        4360        4370        4380        4390        4400

          4300              4310       4320       4330       4340
naip-o TC-GTTACAGCAGCG------GTGGTAACTTTGAGACACCTTCAAAAAGAGCAC------
          : ::  :::  :       ::  : :      :::::  : ::::     :: ::
naip.s GATGATATTGCATTGCTTAATGTCATGAAAGAAAGACATCCTCAATCTAAGTACTTAACT
          4410        4420        4430        4440        4450        4460

              4350        4360        4370        4380              4390
naip-o ---CTGCA--AAGGGA-AGAGCAGGAAGGTCAAAGAGAACAGAGC---AAGAT-CA-CTA
         :: ::  :: ::: :  :: :      :: ::      ::::      ::::: :: :::
naip.s ATTCTCCAGAAATGGATACTGCCGTTCTCTCCAATCATTCAGAAATAAAAGATTCAGCTA
          4470        4480        4490        4500        4510        4520

            4400        4410        4420        4430        4440
naip-o TGAGA--CAGACTACACAACTGGCGGCGAGTCCTGT-GATGAGCTGGAGGAGGAC-TGGA
          :    : :: :  : :: : :    : : : : : :: ::  :  :  : :    : ::
naip.s AAAACTGCTGAATCAATAATTTGTCTTGGGGCATATTGAGGATGTAAAAAAAGTTGTTGA
          4530        4540        4550        4560        4570        4580

      4450                  4460        4470             4480
naip-o TCAGGG-----------AATATCCACC--TATCACTTCAGAT----CA-ACAAAGACAAC
       : :  :             : ::::::    :::     : : ::     :: :::::   ::
naip.s TTAATGCTAAAAACCAAATTATCCAAAATTATTTTATTAAATATTGCATACAAAAGAAAA
          4590        4600        4610        4620        4630        4640

    4490                        4500       4510       4520       4530
naip-o TGT-----------------ACAAGAGGAATTTTGACACTGGCCTACAGGAATACAAG--
       :::                  :::: :  ::     :::: : ::: ::      :: :
naip.s TGTGTAAGGCTTGCTAAAAAACAAAACAAAACAAAACACAGTCCTGCATACTCACCACCA
          4650        4660        4670        4680        4690        4700
```

Fig. 5I

```
                  4540              4550                        4560
naip-o AGCTTAC------AATCAGAAC--------TTGA------TGAG--ATCAA-------TA
       :::: :       :::::  ::        ::::      ::::  ::: :        ::
naip.s AGCTCAAGAAATAAATCATCACCAATACCTTTGAGGTCCCTGAGTAATCCACCCCAGCTA
         4710       4720       4730       4740       4750       4760

            4570       4580       4590       4600
naip-o AAG---AACTCTCCCGTTTGG---ATAAAGAA-----TTGGATGACTATAGAGAA----G
       :::   ::: :: :  :   :   ::: :: :      :    :: : :: :  ::    :
naip.s AAGGCAAACCCTTCAATCAAGTTTATACAGCAAACCCTCCATTGTCCATGGTCAACAGGG
         4770       4780       4790       4800       4810       4820

          4610       4620       4630       4640         4650         4660
naip-o AAAGTGAAGAGTACATGGCTGCTGCTG-ATGAATA---CAATAGACTGAAGCA--AGTGA
       :: : : :: : ::: : ::::   :  ::  : :   ::  : :  :::: :  : : :
naip.s AAGGGGTTGGGGACAGGTCTGCCAATCTATCTAAAAGCCACAATATGGAAGAAGTATTCA
         4830       4840       4850       4860       4870       4880

            4670       4680       4690       4700
naip-o AGGGATCTGC-AGATTACAAAAGTAA--GAAGAATCA-TTGCAAGCA-----------G
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
naip.s ATTTATATAATAAATGGCTAACTTAACGGTTGAATCACTTTCATACATGGATGAAACGGG
         4890       4900       4910       4920       4930       4940

          4710       4720                  4730                  4740
naip-o TTAAACAGCAAATTGTCACACATC-----------AAGAAGATGGT----------TGGA
       :: ::::    ::   ::   :::           :::: :::: :           :: :
naip.s TTTAACACAGGATCCACATGAATCTTCTGTGGGCCAAGA-GATGTTCCTTAATCCTTGTA
         4950       4960       4970       4980       4990       5000

            4750       4760       4770
naip-o GA---------CTAT---GA--TAG-------ACAGAA-----AACATAGAAGGC--TGA
       ::          ::::   ::  :::       :::: :     ::: ::      :  : :
naip.s GAACCTGTTTTCTATATTGAACTAGCTTTGGTACAGTAGAGTTAACTTACTTTCCATTTA
         5010       5020       5030       5040       5050       5060

            4780       4790             4800       4810         4820
naip.o T-----GCCAAGTTGTTTGAGAAA------TTAAGTATC--TGACATCTCTGCAAT--CT
       :     :::::  :      ::::       ::: : :    :::: ::  : ::     ::
naip.s TCCACTGCCAATATAAAGAGGAAACAGGGGTTAGGGAAAAATGACTTCATTCCAGAGGCT
         5070       5080       5090       5100       5110       5120

            4830       4840       4850       4860       4870
naip-o TCTCAGAAGGCAA---ATG----ACTTTGGACCATAACCCCGGAAGCCAAACCTCTGTGA
       :::::::   :::   :::    : ::: ::   :  :    ::: ::: :  :: ::
naip.s TCTCAGAGTTCAACATATGCTATAATTTAGAATTTT-CTTATGAATCCACTCTACT-TGG
         5130       5140       5150       5160       5170       5180

            4880       4890       4900       4910       4920
naip-o GCATCACAGTTTTGGT------TGCTTTAATATCAT--CAGTATTGAAGCATTTTATAA-
       : :  : :  :::  :       :: ::  :::: ::  :  :::    :: :::: :::
naip.s GTAGAAAATATTTTATCTCTAGTGATTGCATATTATTTCCATATCATAGTATTTCATAGT
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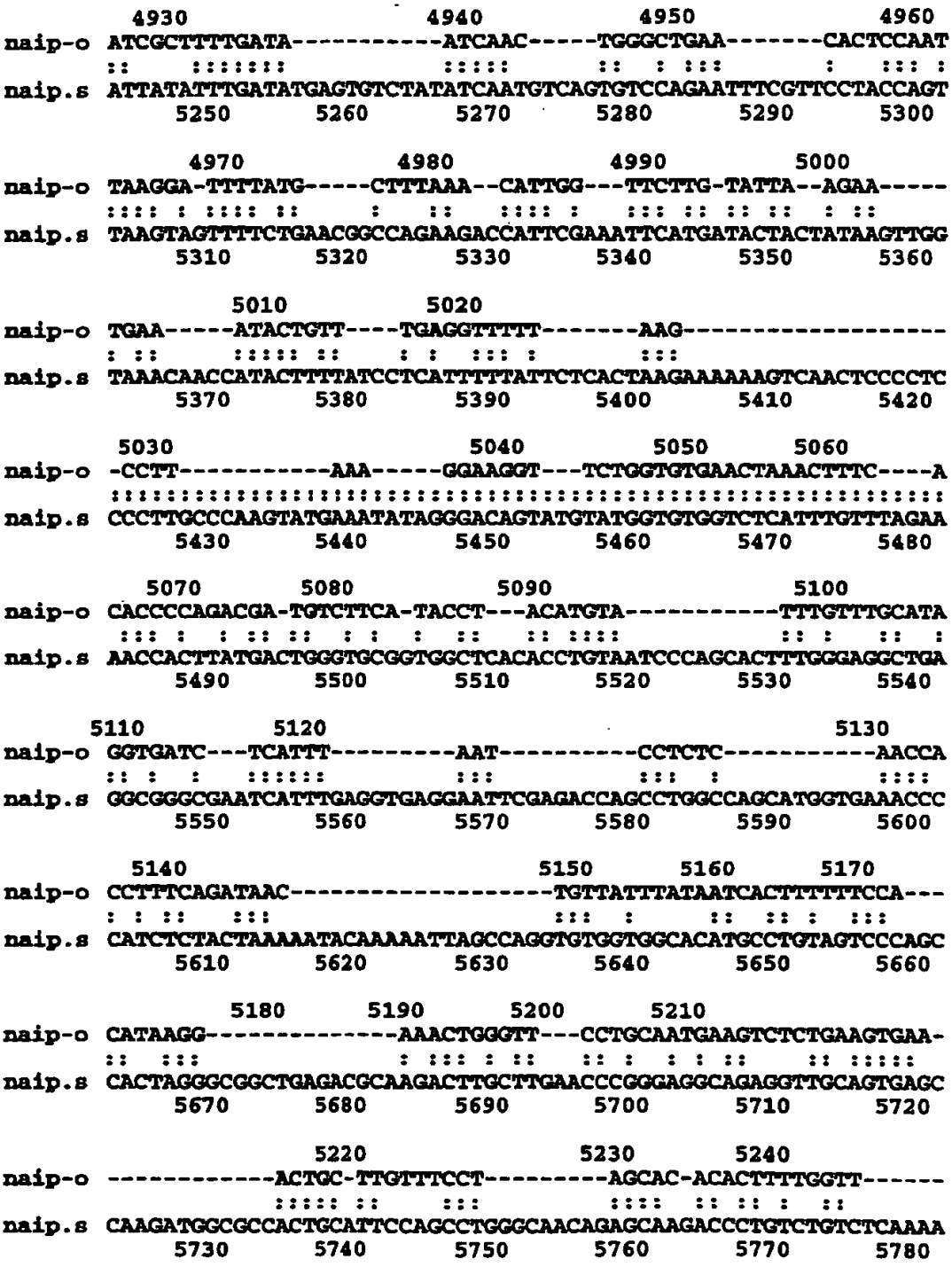
Fig. 5J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4970 4980 4990 5000
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5310 5320 5330 5340 5350 5360
5010 5020
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5430 5440 5450 5460 5470 5480
5070 5080 5090 5100
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naip.s AACCACTTATGACTGGGTGCGGTGGCTCACACCTGTAATCCCAGCACTTTGGGAGGCTGA
5490 5500 5510 5520 5530 5540
5110 5120 5130
naip-o GGTGATC---TCATTT---------AAT----------CCTCTC----------AACCA
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5550 5560 5570 5580 5590 5600
5140 5150 5160 5170
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5670 5680 5690 5700 5710 5720
5220 5230 5240
naip-o ----------ACTGC-TTGTTTCCT---------AGCAC-ACACTTTTGGTT------
naip.s CAAGATGGCGCCACTGCATTCCAGCCTGGGCAACAGAGCAAGACCCTGTCTGTCTCAAAA
5730 5740 5750 5760 5770 5780

Fig. 5K

```
            5250       5260       5270       5280       5290
naip-o ---------AAGTCTGTTTTATGACTTCATTAATAATAAATTCCGGCATCA--TAC--AG
                ::  :  :: :::  ::   :  :: :  :::: : : ::    :::  ::
naip.s CAAAAAACAAAACCACTTATATTGCTAGCTACATTAAGAATTTCTGAATATGTTACTGAG
            5790       5800       5810       5820       5830       5840

            5300                  5310       5320       5330
naip-o CTA-CTCCTC----CC--------TACCGCCACCTCCACAGACACCACTCTCCTGGT---
       ::  ::  :      ::        :: :   :  :  :   ::::::    : :: :
naip.s CTTGCTTGTGGTAACCATTTATAATATCAGAAAGTATATGTACACCAAAA-CATGTTGAA
            5850       5860       5870       5880       5890       5900

          5340       5350                            5360
naip-o --TCCATCTCCT-CTGCTGC-------------------TTCTAGCTCC------CTGC
         :::::  :   : :  :::                    :: :: ::         :::
naip.s CATCCATGTTGTACAACTGAAATATAAATAATTTTGTCAATTATACCTAAATAAAACTGG
            5910       5920       5930       5940       5950       5960

               5370                  5380       5390       5400
naip-o ---------TCTGGC--TTCA---------AGGTGCGCAGGACCTGCTTCCTTG--GTGA
               :::::   :: :          : ::     ::   : : : ::    :  :::
naip.s AAAAAAATTTCTGGAAGTTTATATCTAAAAATGTTAATAGTGCGTACCTCTAGGAAGTGG
            5970       5980       5990       6000       6010       6020

         5410       5420       5430       5440          5450       5460
naip-o TCCTCTGTAGTCTCCCACACCCCACATTATCTACAAA-CTGA--TGACTCCTAATTTACA
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naip.s GCCTG-GAAGCCATTCTTACTTTTCAGTCTCTCCCATTCTGTACTGTTTTTTGTTTTACT
            6030       6040       6050       6060       6070

            5470       5480       5490                  5500
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       :     :: :: : :    :::    : :: :  ::    :    ::       :::
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Fig. 5L

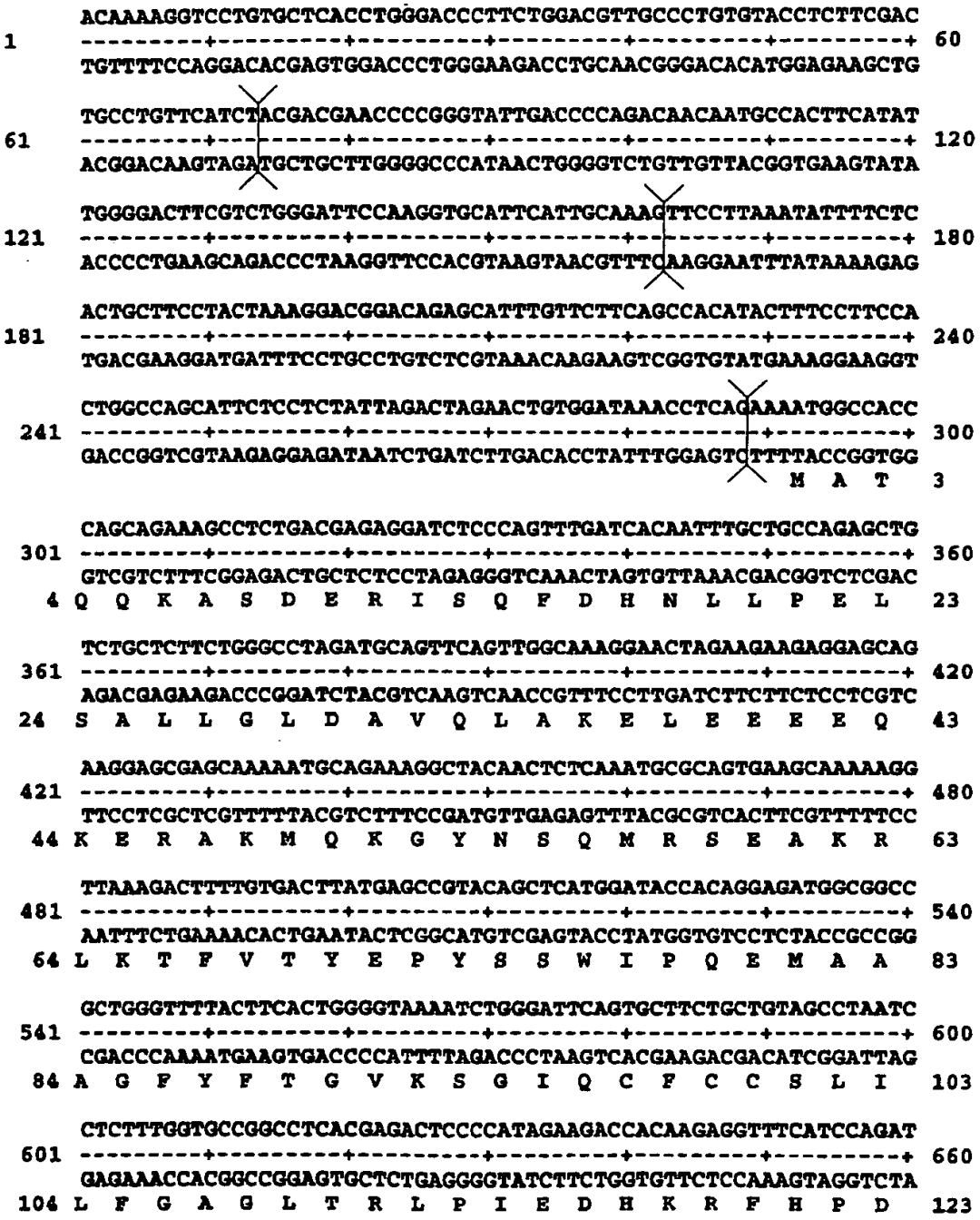
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Fig. 6A

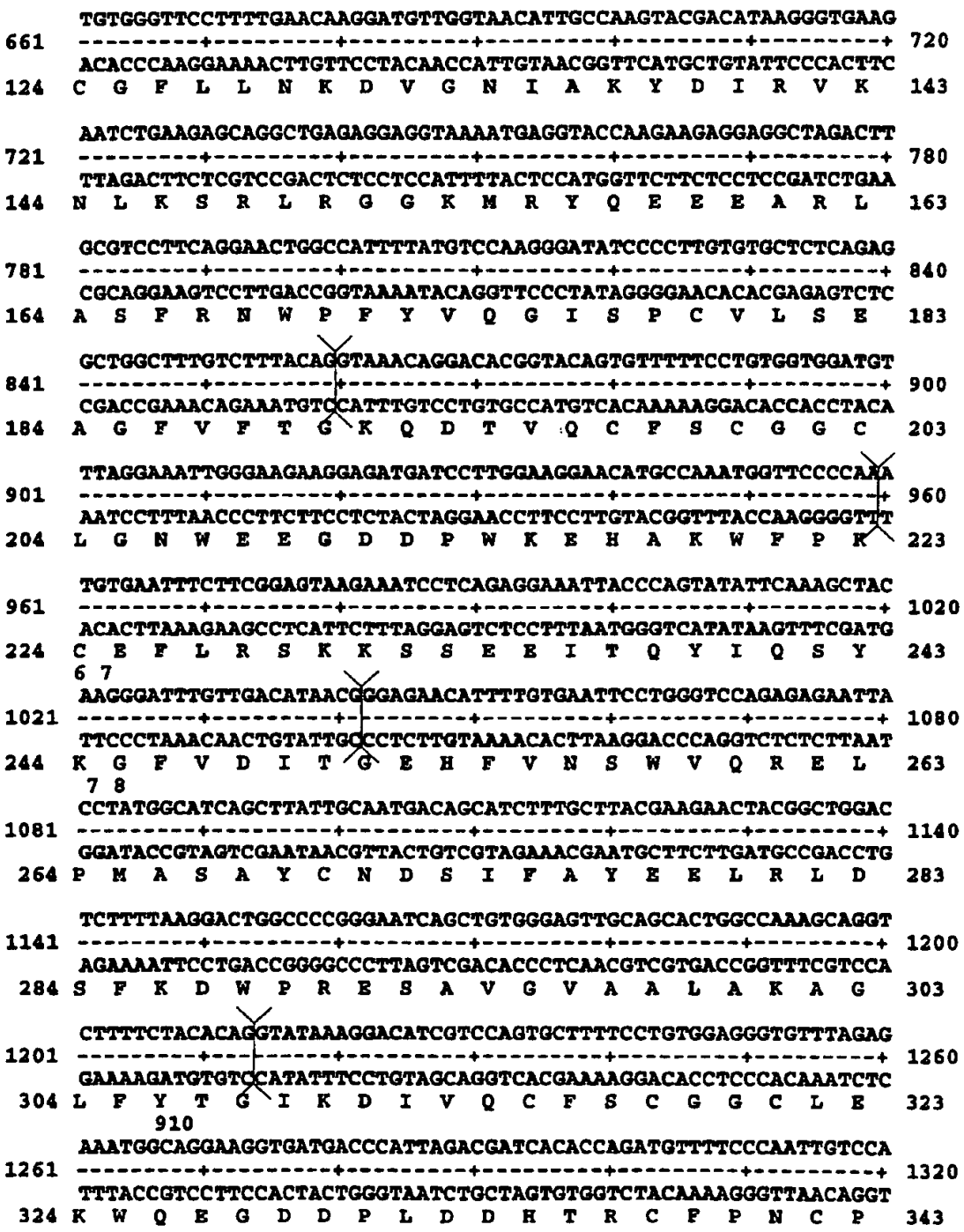
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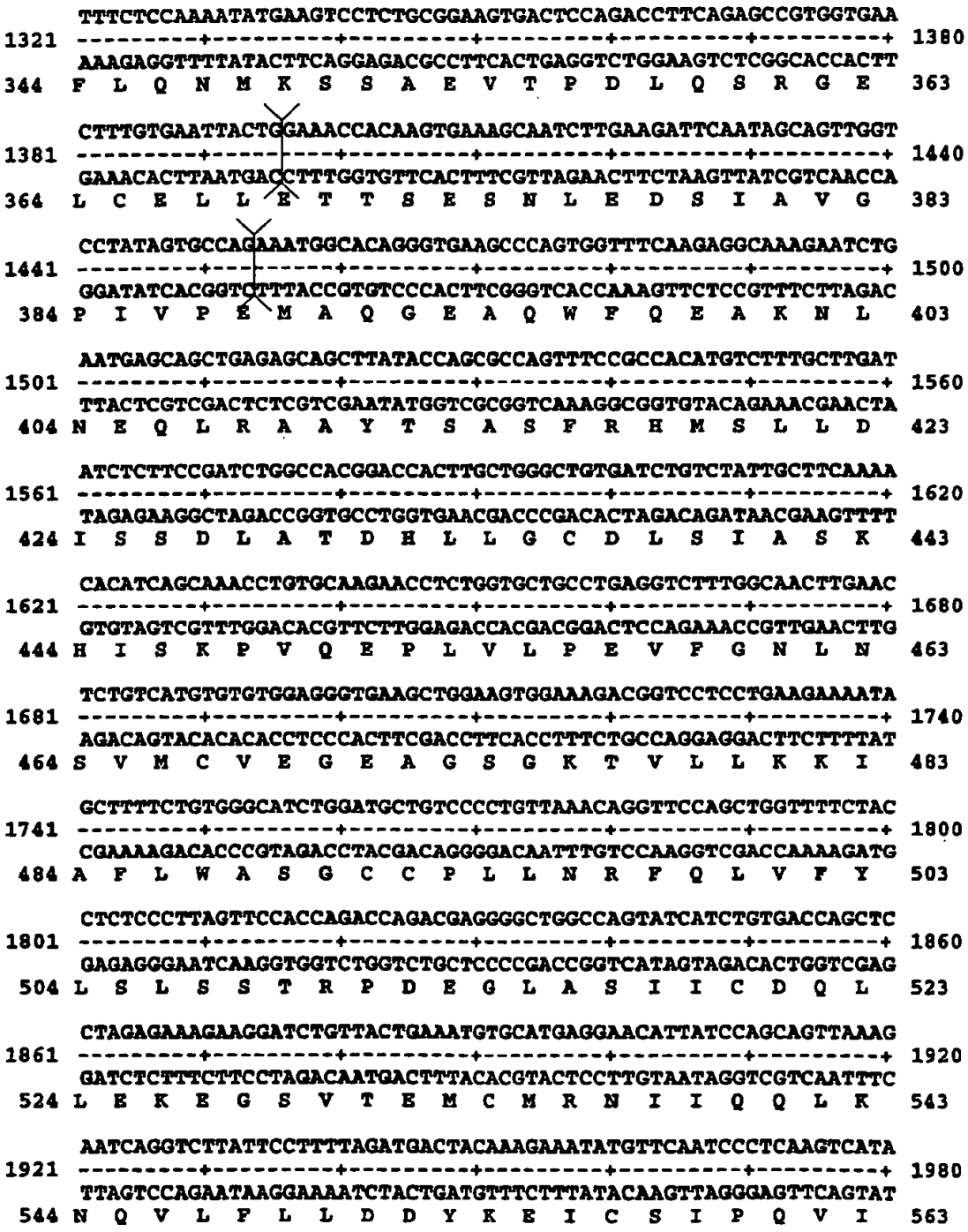
Fig. 6B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524 L E K E G S V T E M C M R N I I Q Q L K 543
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1921 1980
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544 N Q V L F L L D D Y K E I C S I P Q V I 563

Fig. 6C

```
      GGAAAACTGATTCAAAAAAACCACTTATCCCGGACCTGCCTATTGATTGCTGTCCGTACA
1981  ---------+---------+---------+---------+---------+---------+  2040
      CCTTTTGACTAAGTTTTTTTGGTGAATAGGGCCTGGACGGATAACTAACGAC AGGCATGT
 564  G  K  L  I  Q  K  N  H  L  S  R  T  C  L  L  I  A  V  R  T    583

      AACAGGGCCAGGGACATCCGCCGATACCTAGAGACCATTCTAGAGATCAAAGCATTTCCC
2041  ---------+---------+---------+---------+---------+---------+  2100
      TTGTCCCGGTCCCTGTAGGCGGCTATGGATCTCTGGTAAGATCTCTAGTTTCGTAAAGGG
 584  N  R  A  R  D  I  R  R  Y  L  E  T  I  L  E  I  K  A  F  P    603

      TTTTATAATACTGTCTGTATATTACGGAAGCTCTTTTCACATAATATGACTCGTCTGCGA
2101  ---------+---------+---------+---------+---------+---------+  2160
      AAAATATTATGACAGACATATAATGCCTTCGAGAAAAGTGTATTATACTGAGCAGACGCT
 604  F  Y  N  T  V  C  I  L  R  K  L  F  S  H  N  M  T  R  L  R    623

      AAGTTTATGGTTTACTTTGGAAAGAACCAAAGTTTGCAGAAGATACAGAAAACTCCTCTC
2161  ---------+---------+---------+---------+---------+---------+  2220
      TTCAAATACCAAATGAAACCTTTCTTGGTTTCAAACGTCTTCTATGTCTTTTGAGGAGAG
 624  K  F  M  V  Y  F  G  K  N  Q  S  L  Q  K  I  Q  K  T  P  L    643

      TTTGTGGCGGCGATCTGTGCTCATTGGTTTCAGTATCCTTTTGACCCATCCTTTGATGAT
2221  ---------+---------+---------+---------+---------+---------+  2280
      AAACACCGCCGCTAGACACGAGTAACCAAAGTCATAGGAAAACTGGGTAGGAAACTACTA
 644  F  V  A  A  I  C  A  H  W  F  Q  Y  P  F  D  P  S  F  D  D    663

      GTGGCTGTTTTCAAGTCCTATATGGAACGCCTTTCCTTAAGGAACAAAGCGACAGCTGAA
2281  ---------+---------+---------+---------+---------+---------+  2340
      CACCGACAAAAGTTCAGGATATACCTTGCGGAAAGGAATTCCTTGTTTCGCTGTCGACTT
 664  V  A  V  F  K  S  Y  M  E  R  L  S  L  R  N  K  A  T  A  E    683

      ATTCTCAAAGCAACTGTGTCCTCCTGTGGTGAGCTGGCCTTGAAAGGGTTTTTTTCATGT
2341  ---------+---------+---------+---------+---------+---------+  2400
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 684  I  L  K  A  T  V  S  S  C  G  E  L  A  L  K  G  F  F  S  C    703

      TGCTTTGAGTTTAATGATGATGATCTCGCAGAAGCAGGGGTTGATGAAGATGAAGATCTA
2401  ---------+---------+---------+---------+---------+---------+  2460
      ACGAAACTCAAATTACTACTACTAGAGCGTCTTCGTCCCCAACTACTTCTACTTCTAGAT
 704  C  F  E  F  N  D  D  D  L  A  E  A  G  V  D  E  D  E  D  L    723

      ACCATGTGCTTGATGAGCAAATTTACAGCCCAGAGACTAAGACCATTCTACCGGTTTTTA
2461  ---------+---------+---------+---------+---------+---------+  2520
      TGGTACACGAACTACTCGTTTAAATGTCGGGTCTCTGATTCTGGTAAGATGGCCAAAAAT
 724  T  M  C  L  M  S  K  F  T  A  Q  R  L  R  P  F  Y  R  F  L    743

      AGTCCTGCCTTCCAAGAATTTCTTGCGGGGATGAGGCTGATTGAACTCCTGGATTCAGAT
2521  ---------+---------+---------+---------+---------+---------+  2580
      TCAGGACGGAAGGTTCTTAAAGAACGCCCCTACTCCGACTAACTTGAGGACCTAAGTCTA
 744  S  P  A  F  Q  E  F  L  A  G  M  R  L  I  E  L  L  D  S  D    763

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2581  ---------+---------+---------+---------+---------+---------+  2640
      TCCGTCCTTGTAGTTCTAAACCCTGACATAGTAAACTTTGTTTAGTTGAGTGGGTACTAC
 764  R  Q  E  H  Q  D  L  G  L  Y  H  L  K  Q  I  N  S  P  M  M    783
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Fig. 6D

```
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2641  ---------+---------+---------+---------+---------+---------+  2700
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 784  T  V  S  A  Y  N  N  F  L  N  Y  V  S  S  L  P  S  T  K  A    803

      GGGCCCAAAATTGTGTCTCATTTGCTCCATTTAGTGGATAACAAAGAGTCATTGGAGAAT
2701  ---------+---------+---------+---------+---------+---------+  2760
      CCCGGGTTTTAACACAGAGTAAACGAGGTAAATCACCTATTGTTTCTCAGTAACCTCTTA
 804  G  P  K  I  V  S  H  L  L  H  L  V  D  N  K  E  S  L  E  N    823

      ATATCTGAAAATGATGACTACTTAAAGCACCAGCCAGAAATTTCACTGCAGATGCAGTTA
2761  ---------+---------+---------+---------+---------+---------+  2820
      TATAGACTTTTACTACTGATGAATTTCGTGGTCGGTCTTTAAAGTGACGTCTACGTCAAT
 824  I  S  E  N  D  D  Y  L  K  H  Q  P  E  I  S  L  Q  M  Q  L    843

      CTTAGGGGATTGTGGCAAATTTGTCCACAAGCTTACTTTTCAATGGTTTCAGAACATTTA
2821  ---------+---------+---------+---------+---------+---------+  2880
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 844  L  R  G  L  W  Q  I  C  P  Q  A  Y  F  S  M  V  S  E  H  L    863

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2881  ---------+---------+---------+---------+---------+---------+  2940
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 864  L  V  L  A  L  K  T  A  Y  Q  S  N  T  V  A  A  C  S  P  F    883

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3001  ---------+---------+---------+---------+---------+---------+  3060
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3061  ---------+---------+---------+---------+---------+---------+  3120
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 924  K  T  S  P  R  A  H  F  S  V  L  E  T  C  F  D  K  S  Q  V    943

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3121  ---------+---------+---------+---------+---------+---------+  3180
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 944  P  T  I  D  Q  D  Y  A  S  A  F  E  P  M  N  E  W  E  R  N    963

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3181  ---------+---------+---------+---------+---------+---------+  3240
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3241  ---------+---------+---------+---------+---------+---------+  3300
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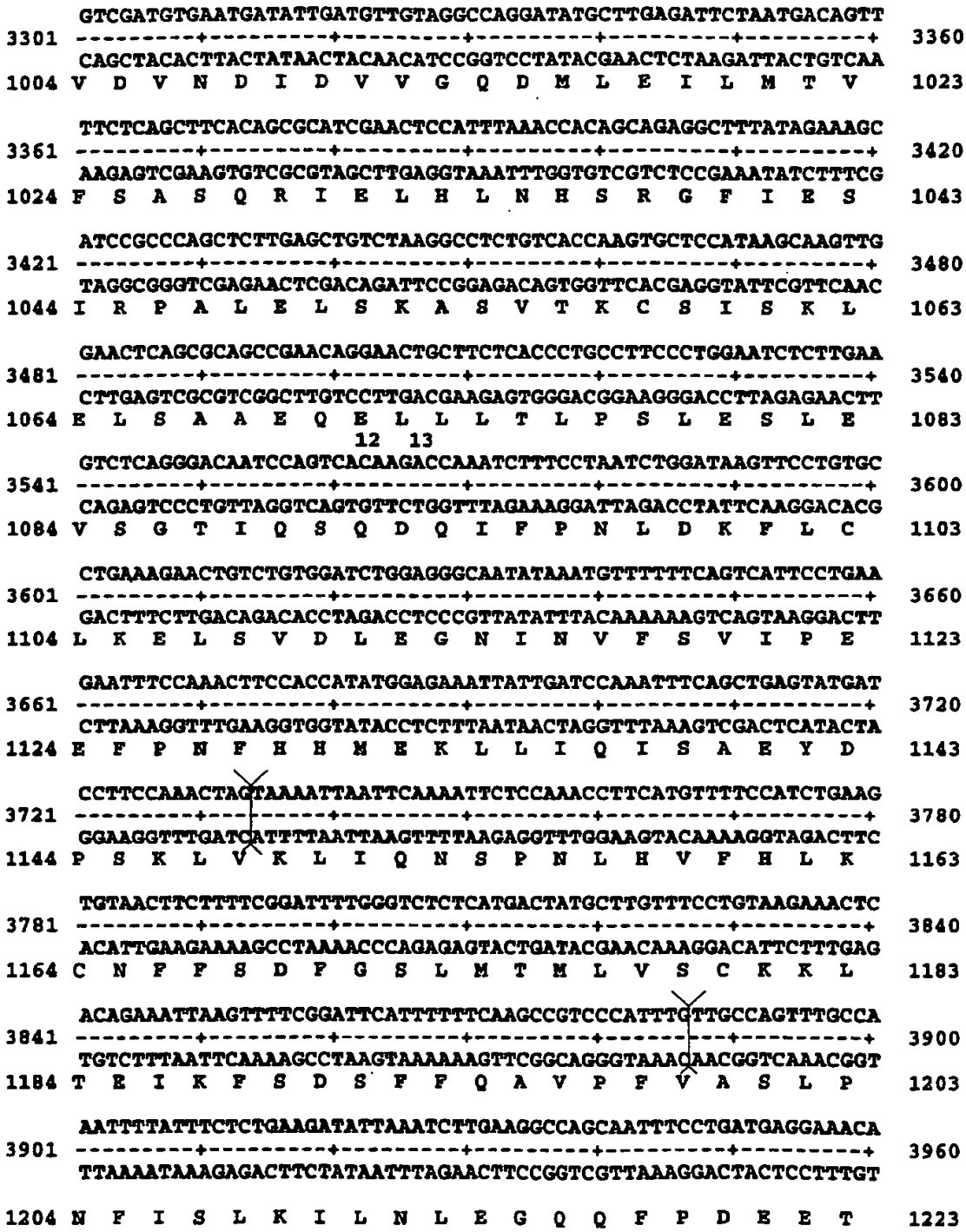
Fig. 6E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3421 3480
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12 13
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3541 3600
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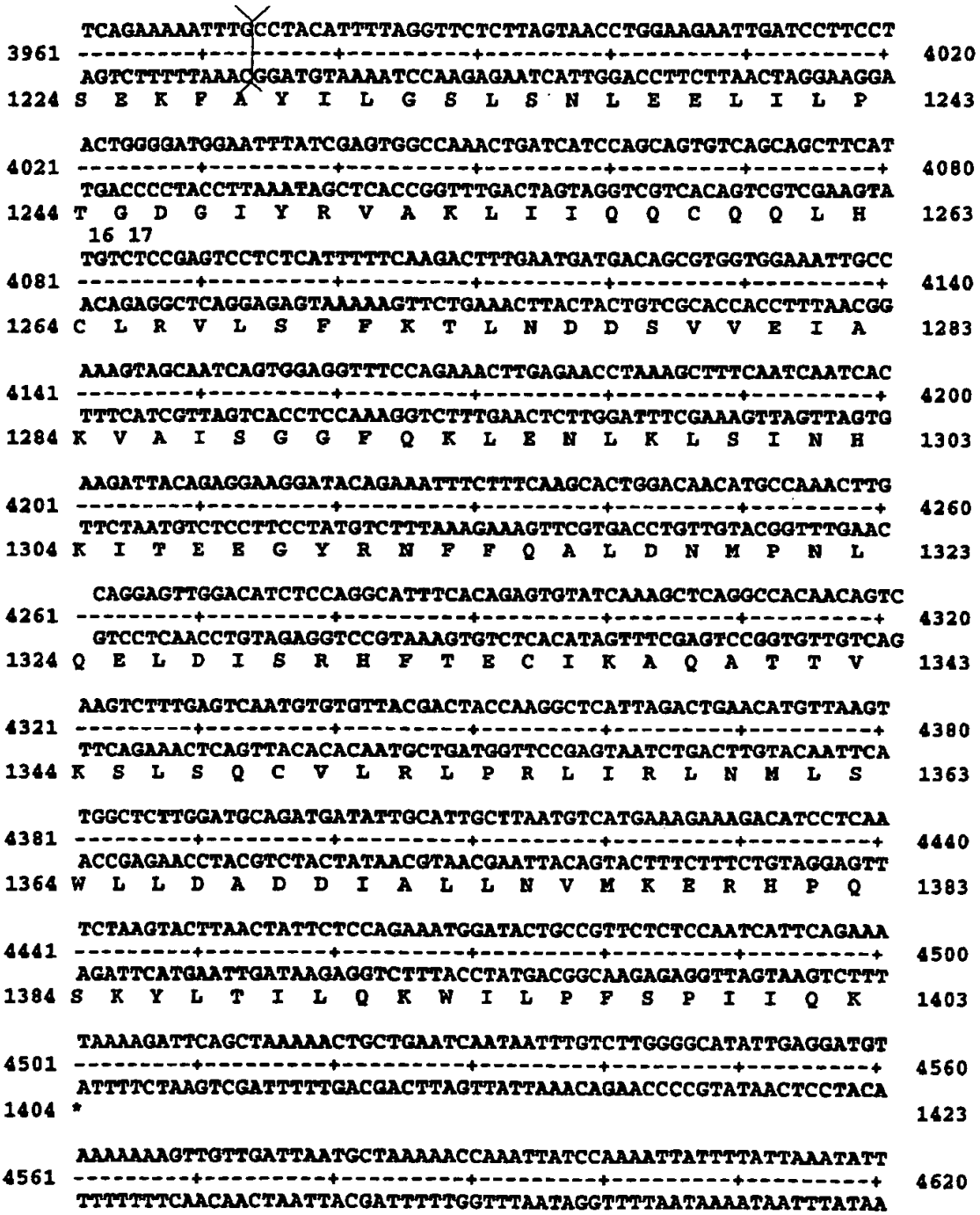
Fig. 6F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4021 4080
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4261 4320
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4561 4620
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Fig. 6G

```
                GCATACAAAAGAAAATGTGTAAGGCTTGCTAAAAAACAAAACAAAACAAAACACAGTCCT
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                CGTATGTTTTCTTTTACACATTCCGAACGATTTTTTGTTTTGTTTTGTTTTGTGTCAGGA

                GCATACTCACCACCAAGCTCAAGAAATAAATCATCACCAATACCTTTGAGGTCCCTGAGT
4681  --------+---------+---------+---------+---------+---------+---------+  4740
                CGTATGAGTGGTGGTTCGAGTTCTTTATTTAGTAGTGGTTATGGAAACTCCAGGGACTCA

                AATCCACCCCAGCTAAAGGCAAACCCTTCAATCAAGTTTATACAGCAAACCCTCCATTGT
4741  --------+---------+---------+---------+---------+---------+---------+  4800
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                CCATGGTCAACAGGGAAGGGGTTGGGGACAGGTCTGCCAATCTATCTAAAAGCCACAATA
4801  --------+---------+---------+---------+---------+---------+---------+  4860
                GGTACCAGTTGTCCCTTCCCCAACCCCTGTCCAGACGGTTAGATAGATTTTCGGTGTTAT

                TGGAAGAAGTATTCAATTTATATAATAAATGGCTAACTTAACGGTTGAATCACTTTCATA
4861  --------+---------+---------+---------+---------+---------+---------+  4920
                ACCTTCTTCATAAGTTAAATATATTATTTACCGATTGAATTGCCAACTTAGTGAAAGTAT

                CATGGATGAAACGGGTTTAACACAGGATCCACATGAATCTTCTGTGGGCCAAGAGATGTT
4921  --------+---------+---------+---------+---------+---------+---------+  4980
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                CCTTAATCCTTGTAGAACCTGTTTTCTATATTGAACTAGCTTTGGTACAGTAGAGTTAAC
4981  --------+---------+---------+---------+---------+---------+---------+  5040
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5041  --------+---------+---------+---------+---------+---------+---------+  5100
                AATGAAAGGTAAATAGGTGACGGTTATATTTCTCCTTTGTCCCCAATCCCTTTTTACTGA

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5101  --------+---------+---------+---------+---------+---------+---------+  5160
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5161  --------+---------+---------+---------+---------+---------+---------+  5220
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5221  --------+---------+---------+---------+---------+---------+---------+  5280
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                GTTCCTACCAGTTAAGTAGTTTTCTGAACGGCCAGAAGACCATTCGAAATTCATGATACT
5281  --------+---------+---------+---------+---------+---------+---------+  5340
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5341  --------+---------+---------+---------+---------+---------+---------+  5400
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Fig. 6H

```
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5461  ---------+---------+---------+---------+---------+---------+  5520
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5521  ---------+---------+---------+---------+---------+---------+  5580
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5581  ---------+---------+---------+---------+---------+---------+  5640
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Fig. 6I

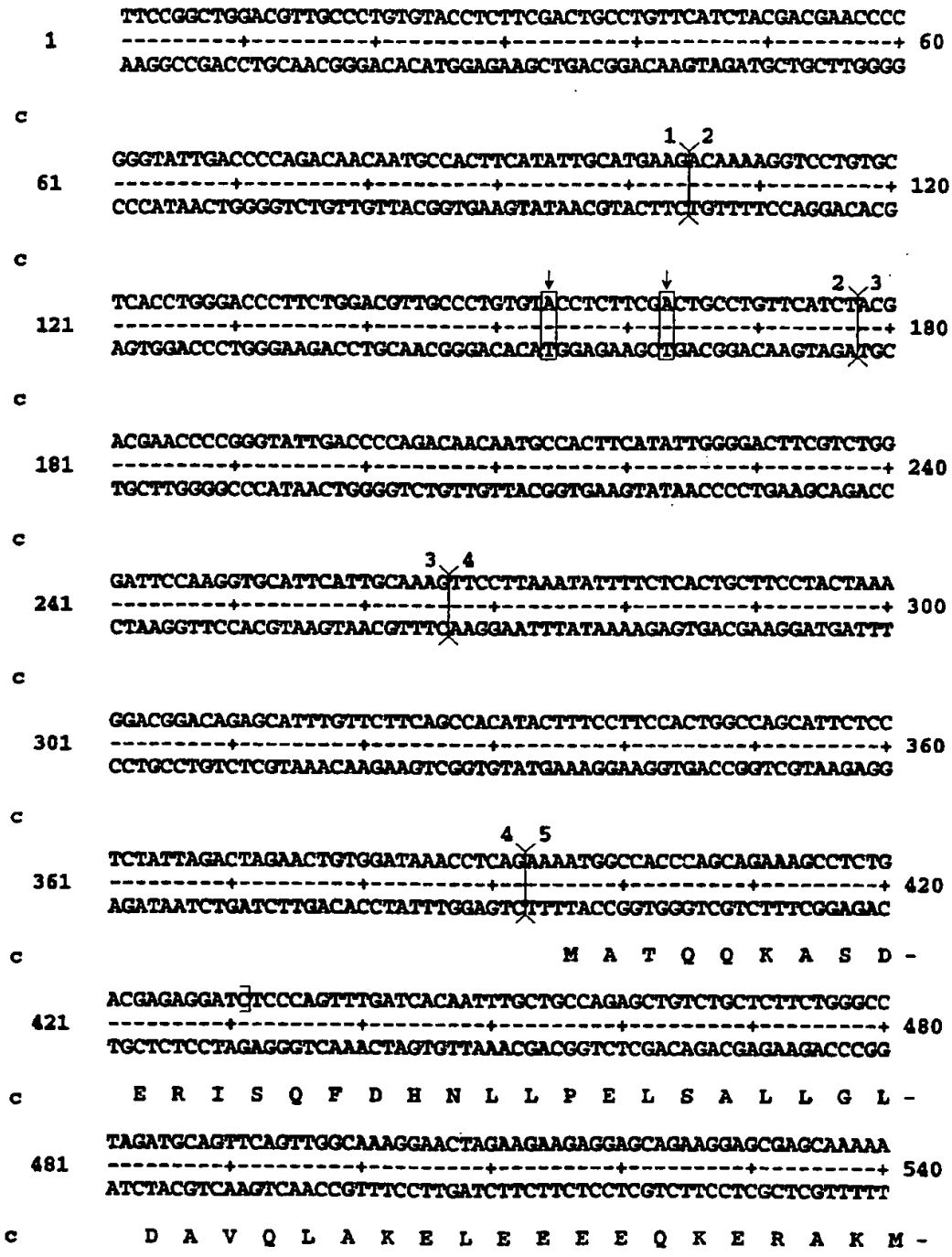
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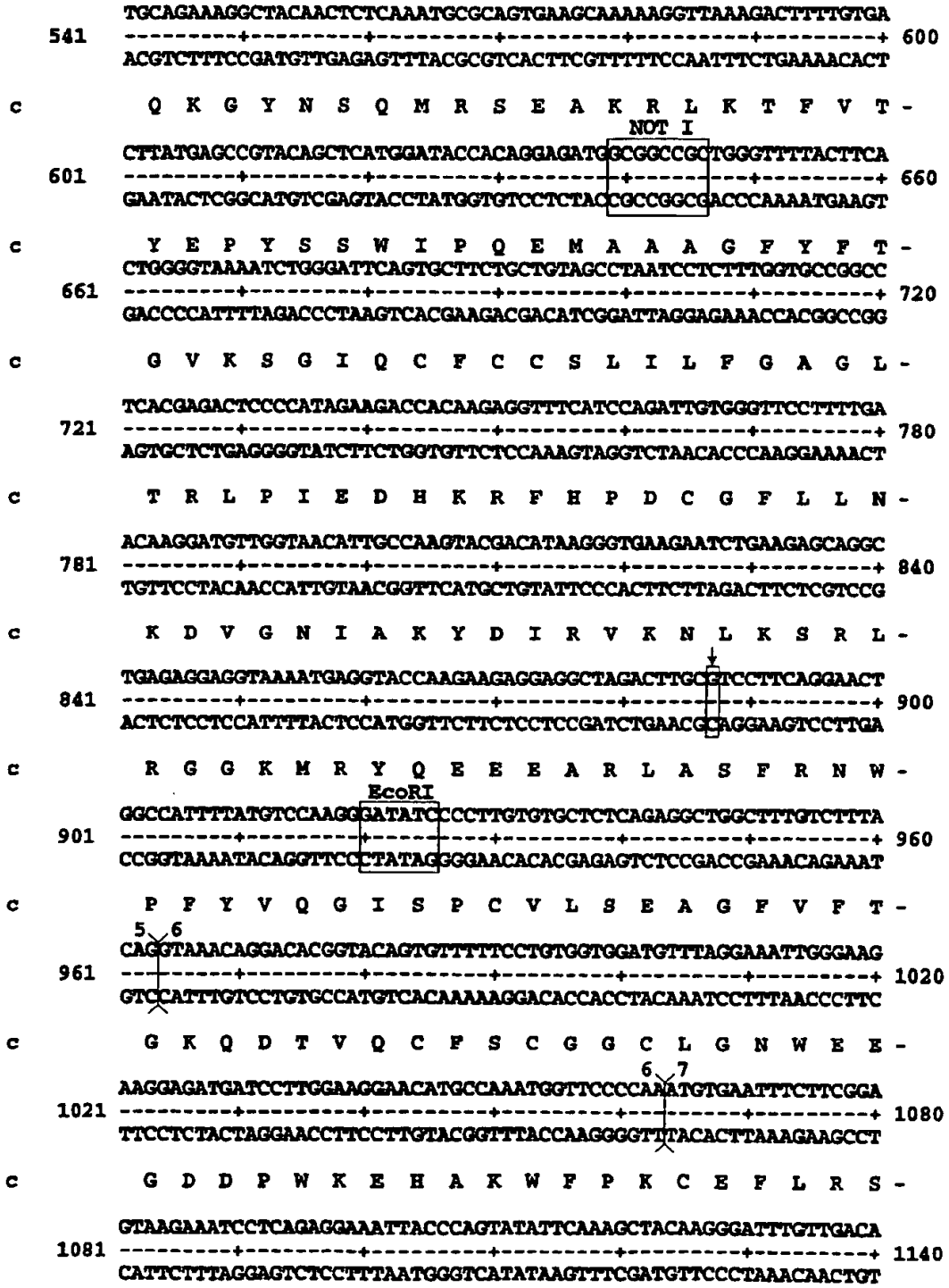
Fig. 7A



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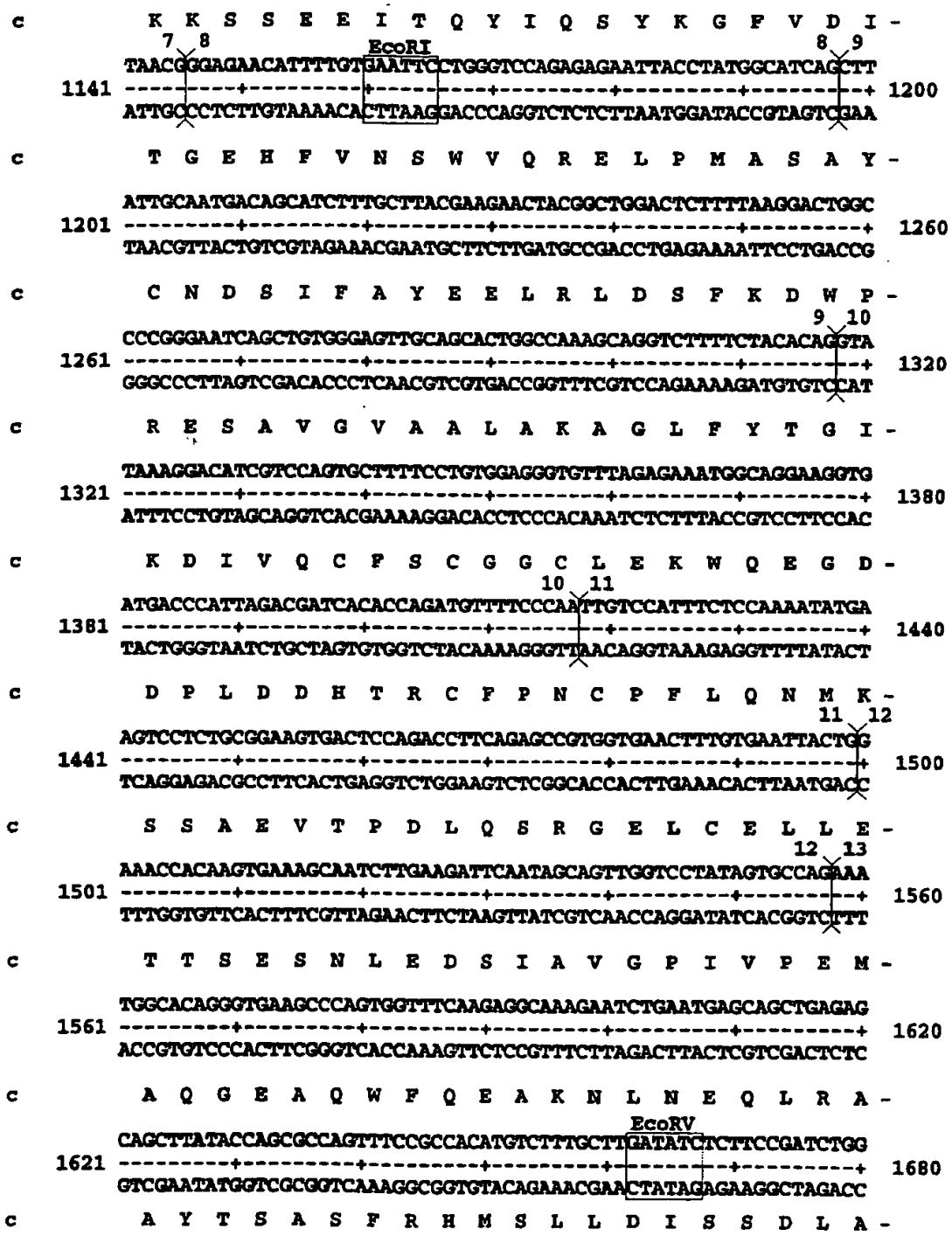
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Fig. 7C

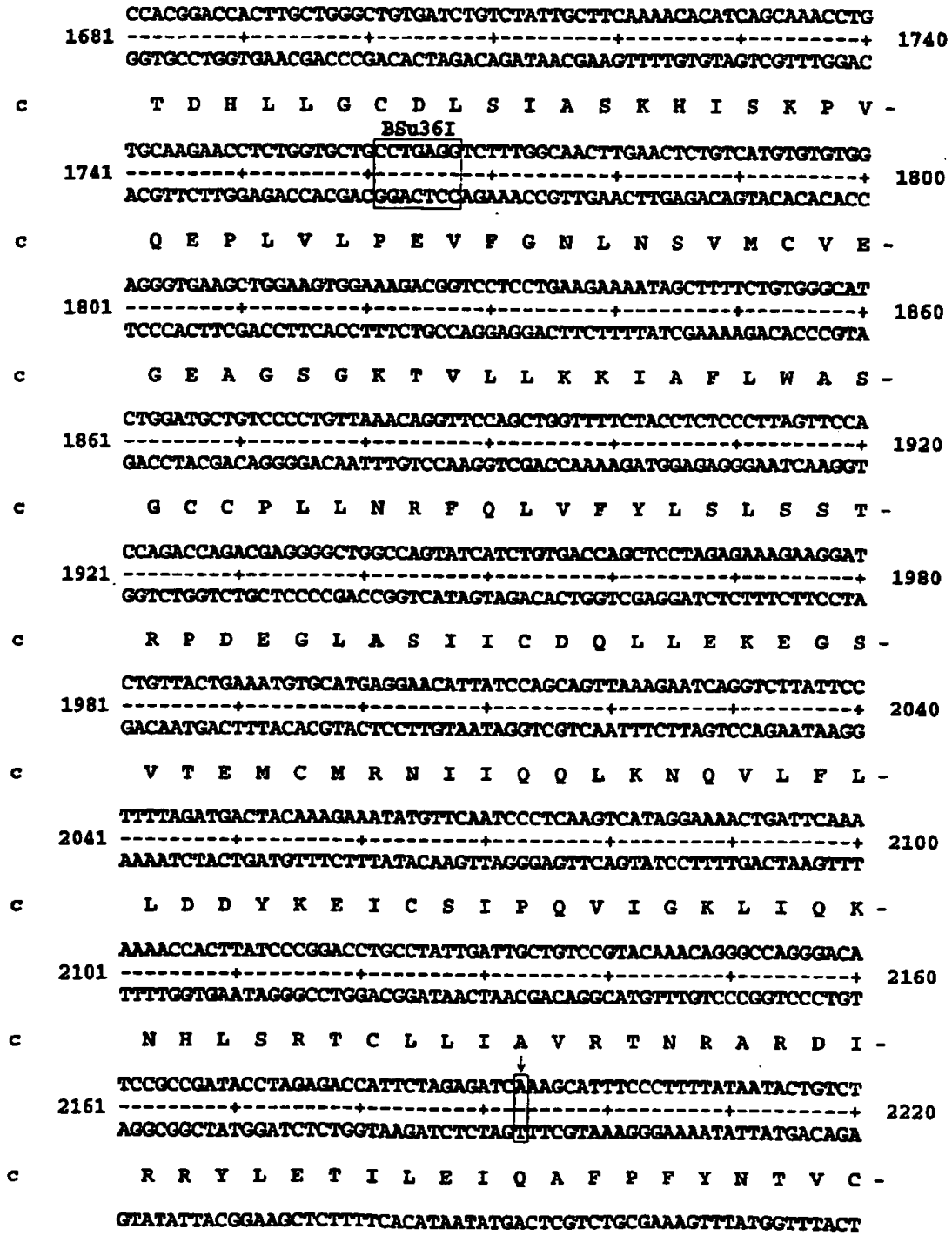

Fig. 7D

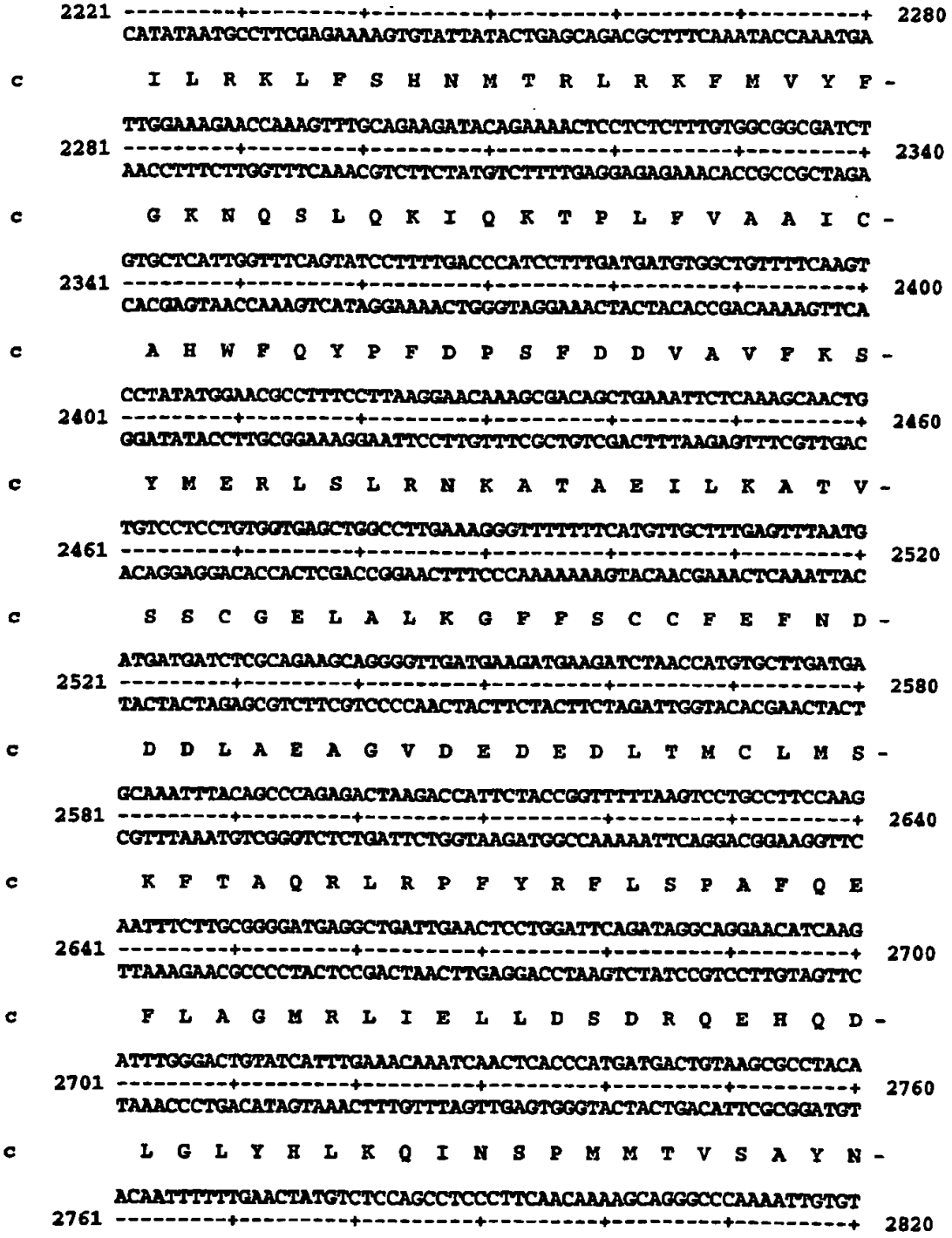
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Fig. 7E

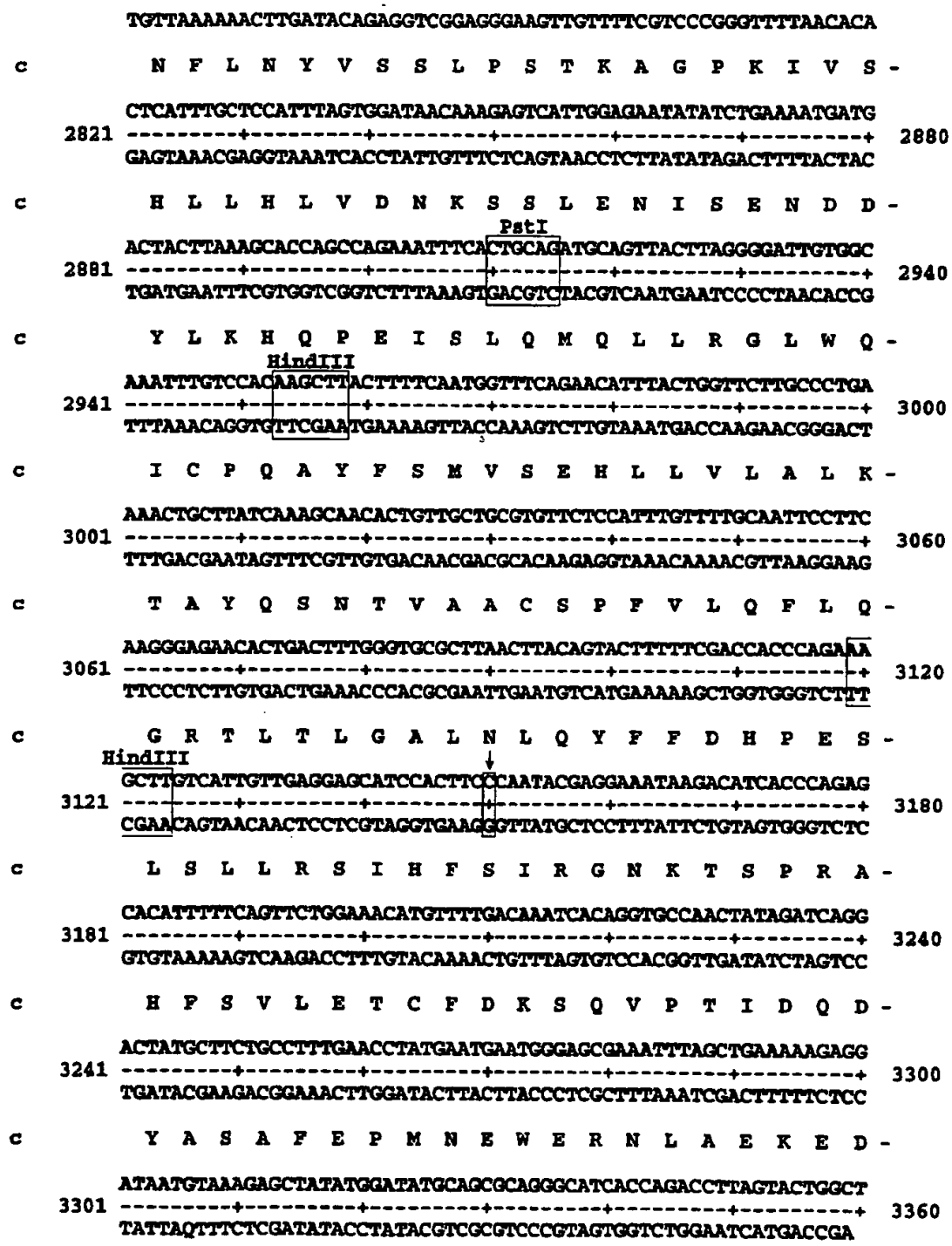
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Fig. 7F

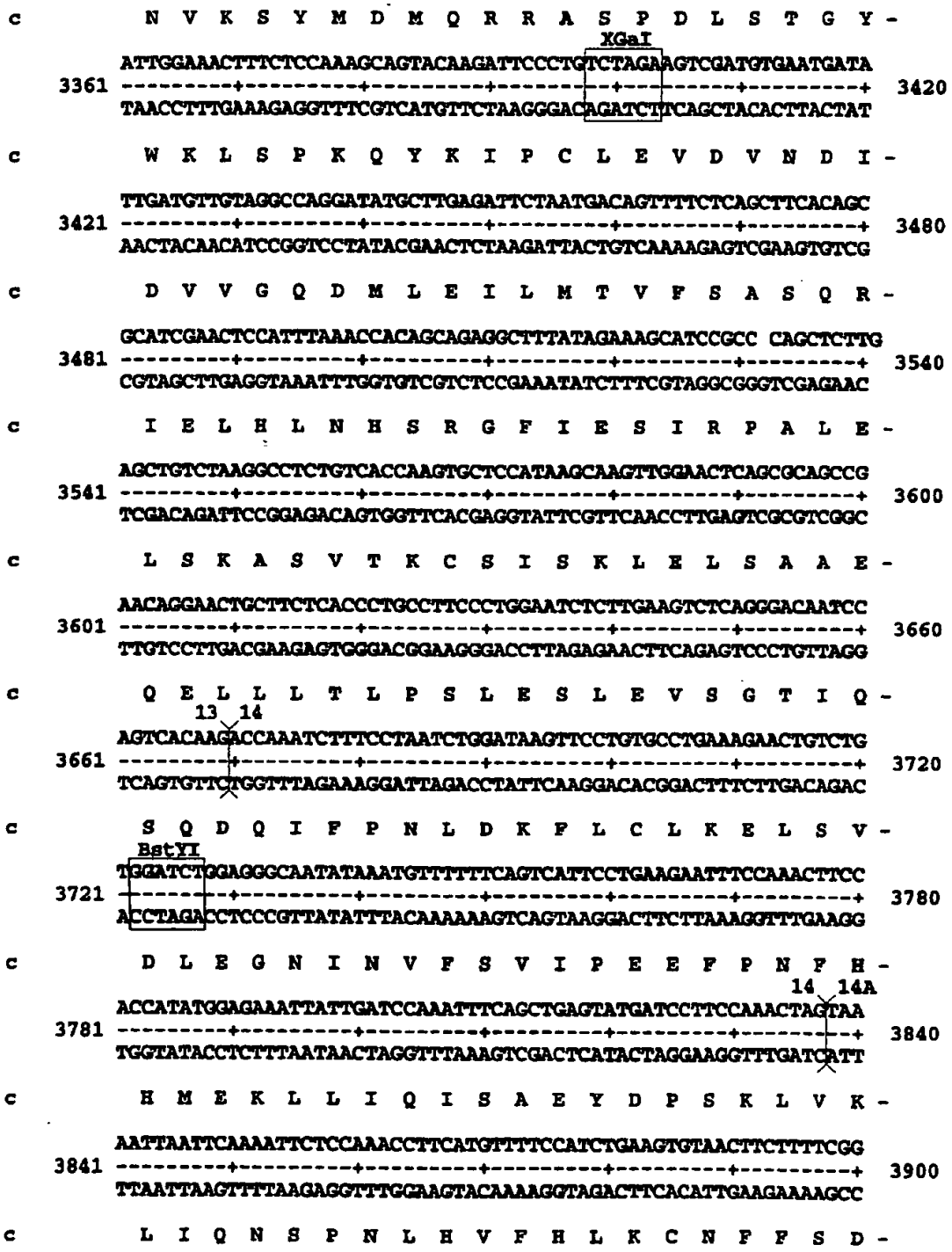
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Fig. 7G

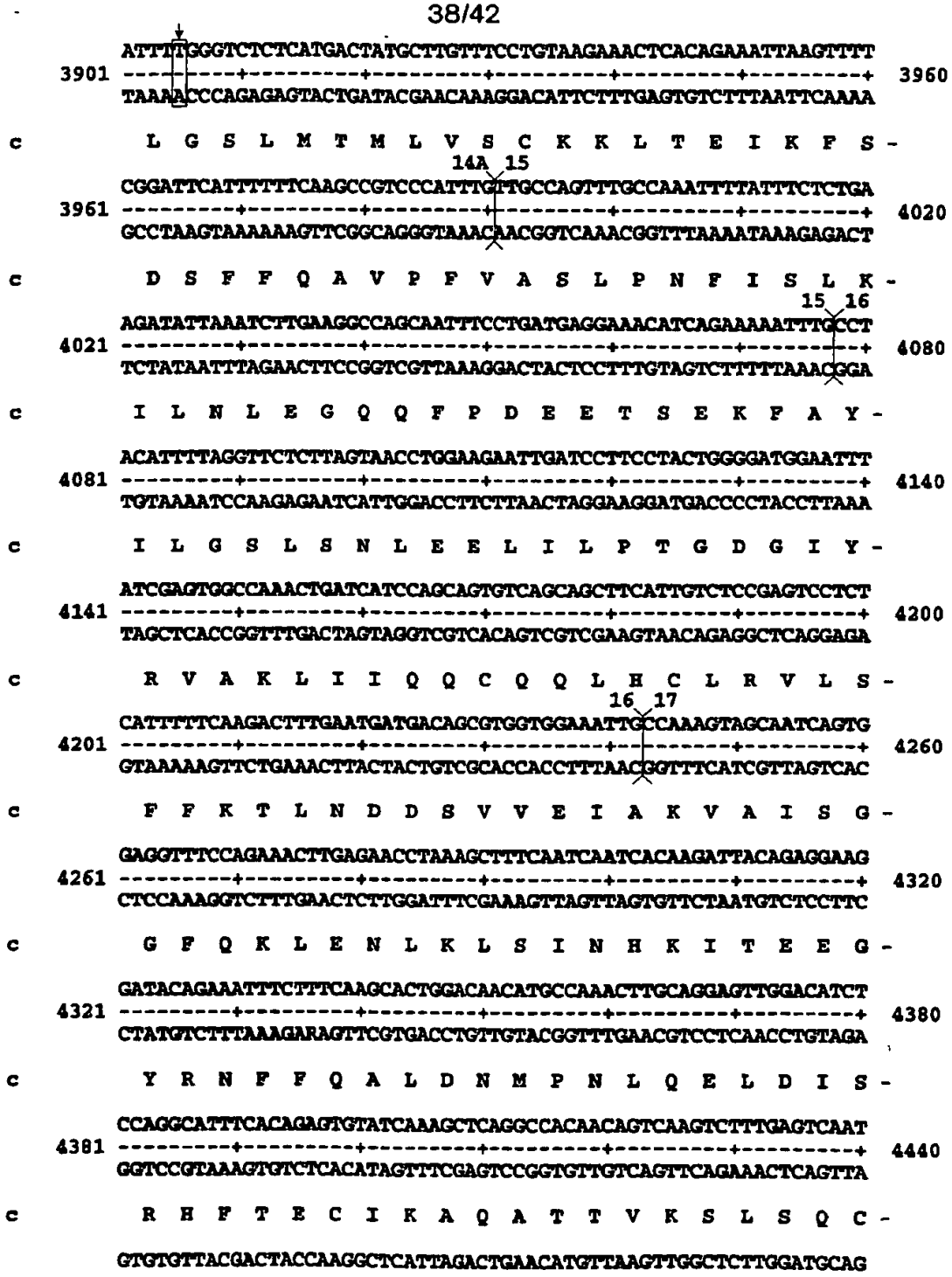
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Fig. 7H

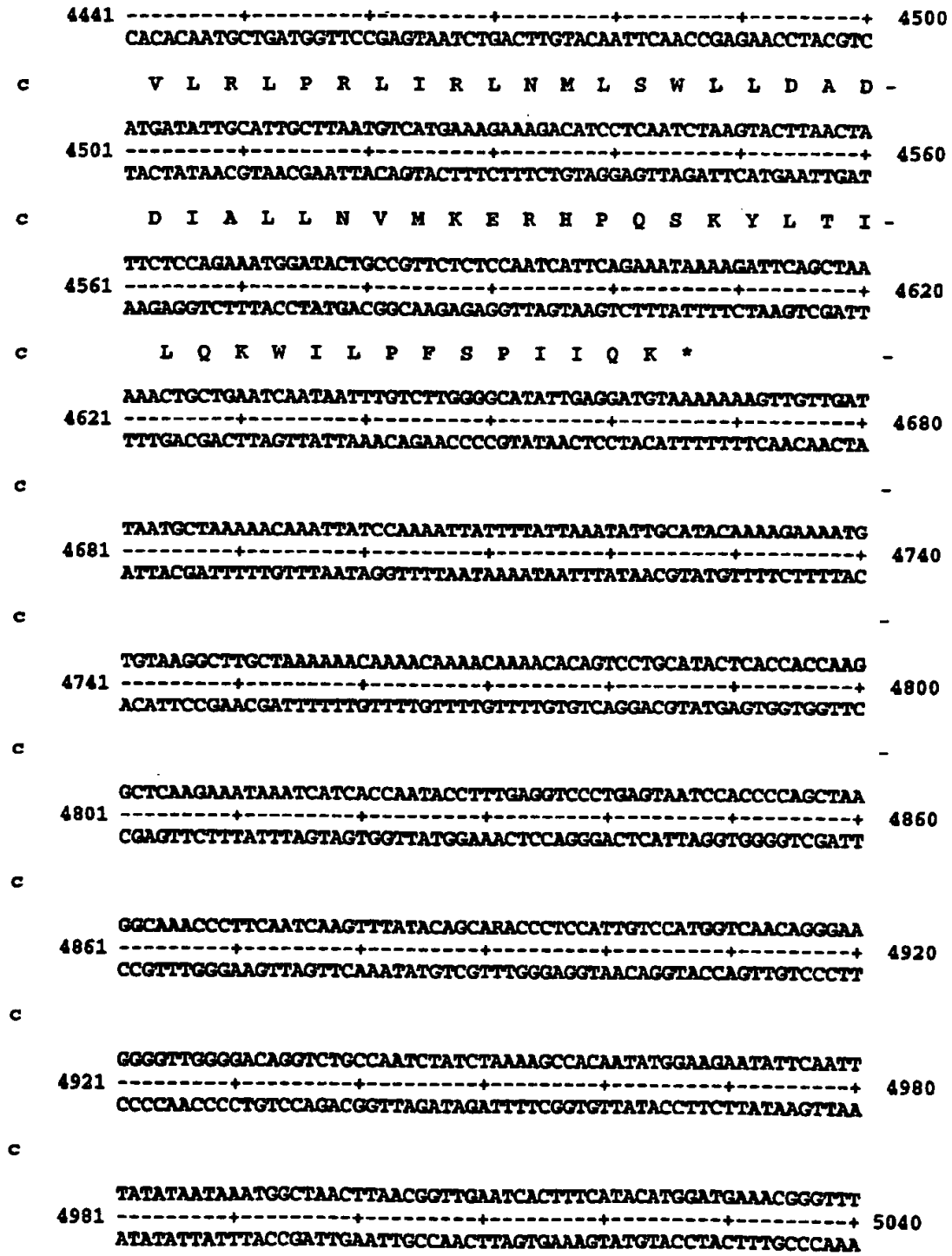
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Fig. 7I

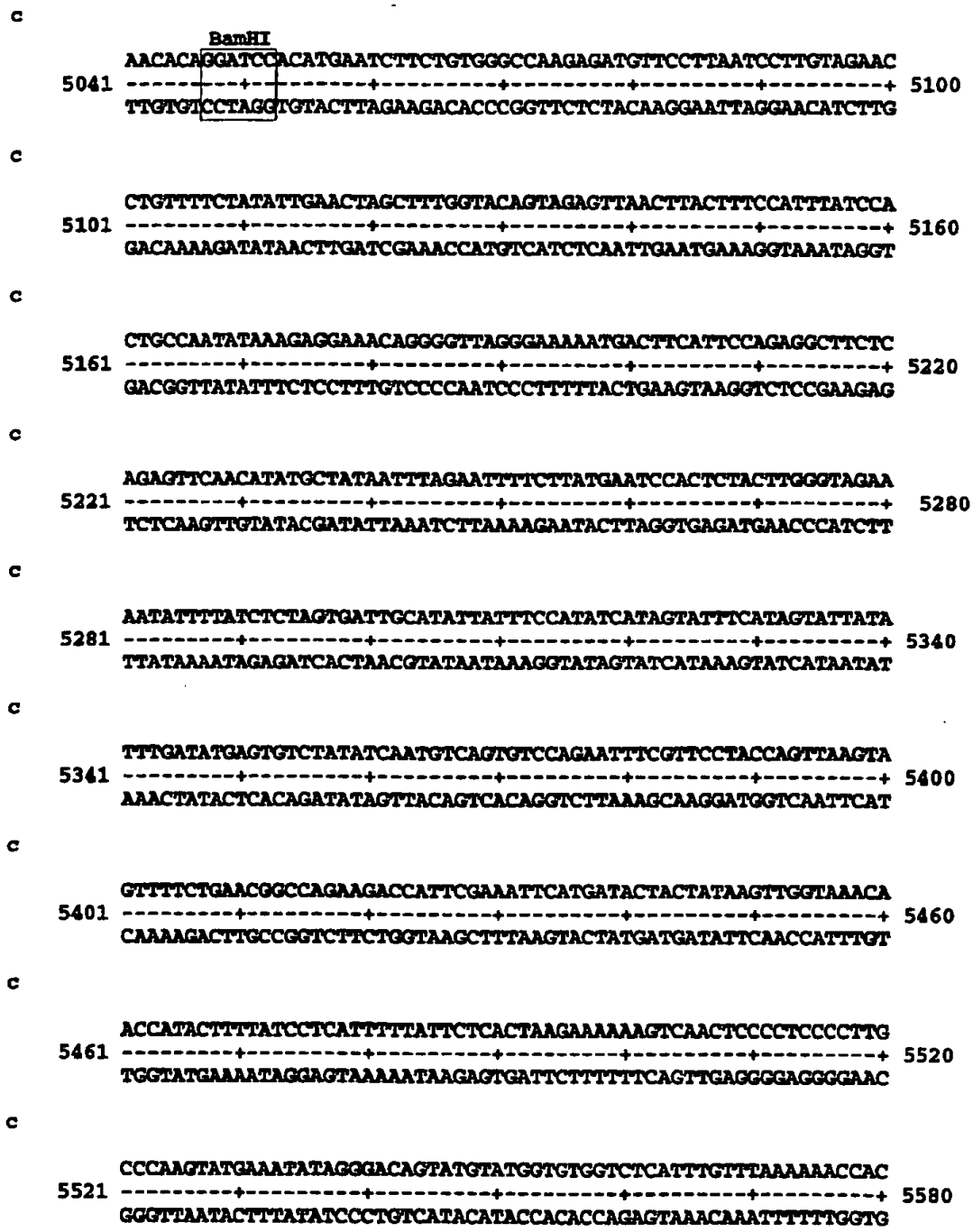
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Fig. 7J

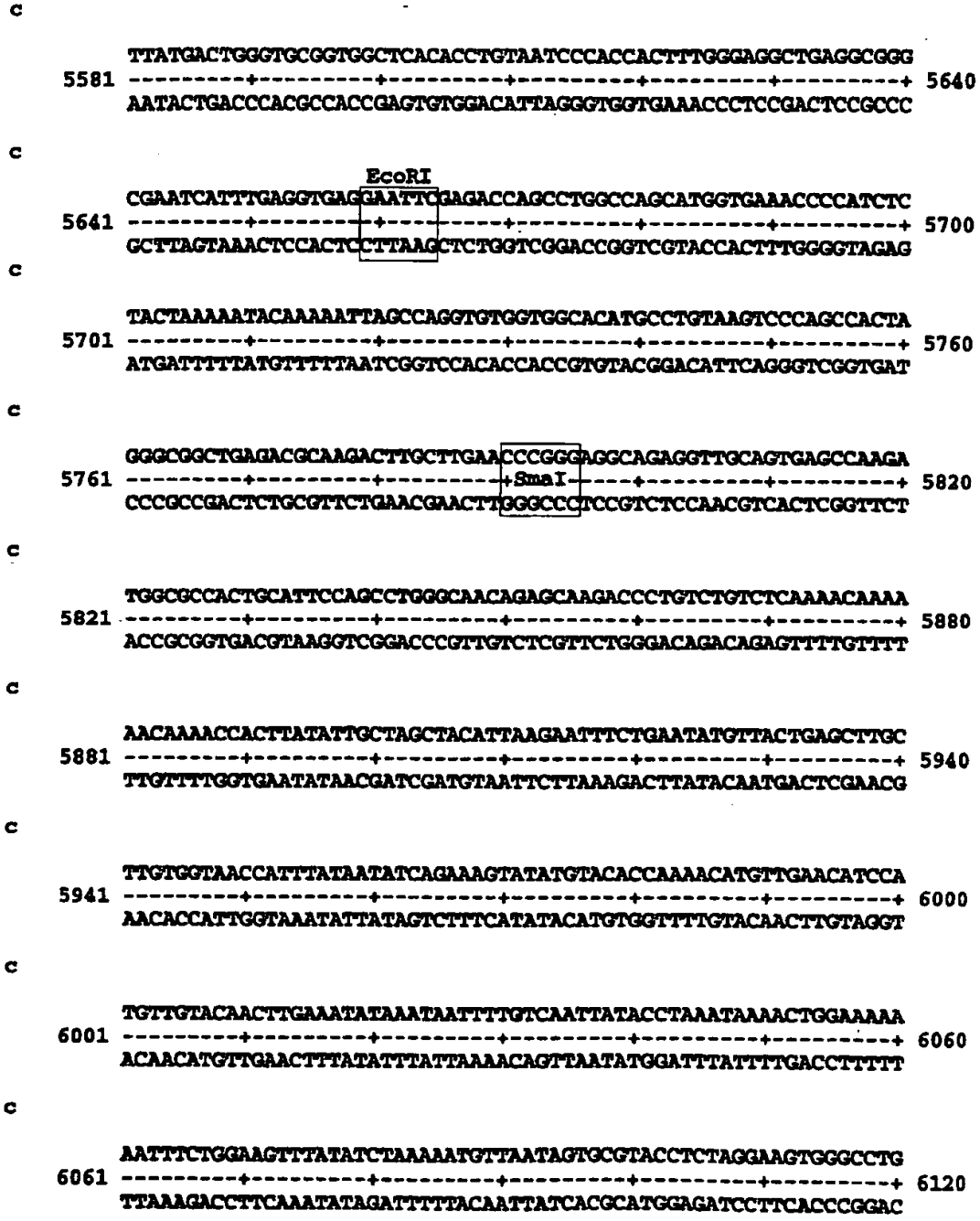
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Fig. 7K

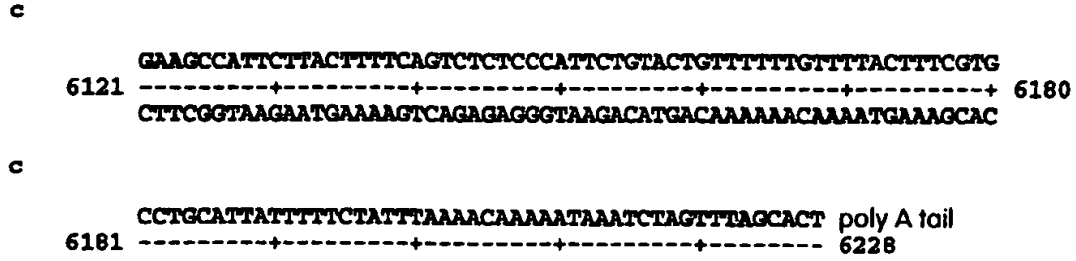
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Fig. 7L